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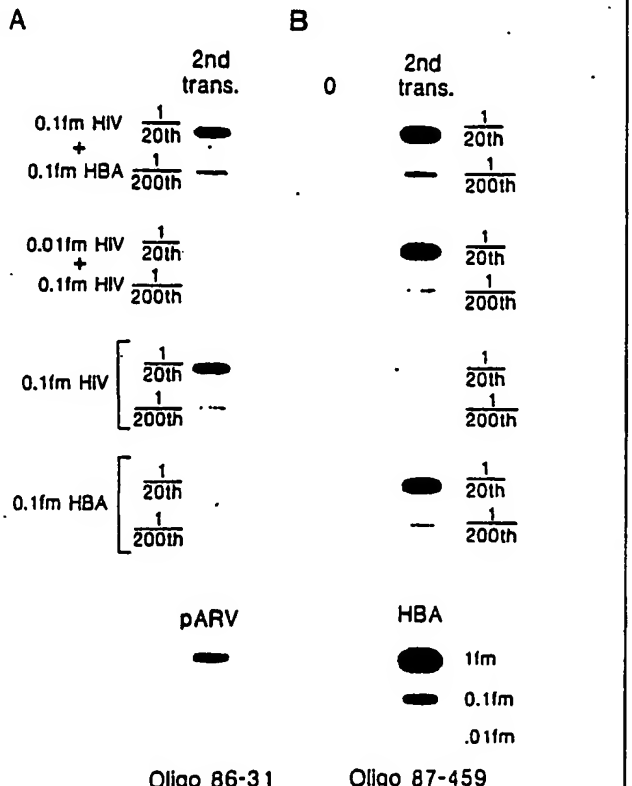
(57) Abstract

The present invention is predicated on the novelty of certain RNA transcripts, their production, optional replication, and use, to achieve desired amplification and detection of corresponding (in sequence) target nucleic acid sequence. The transcripts correspond in sequence to a target nucleic acid sequence contained in an original sample amongst a mixture of nucleic acids, and therefore, the presence of the transcripts in amplified form provides for their detection, and hence by correspondence, the *in vitro* or *x-vivo* detection of the presence of the target nucleic acid sequence in said sample.



TRANSCRIPTION-BASED NUCLEIC ACID AMPLIFICATION/DETECTION SYSTEMS

Description of **WO8810315**

TRANSCRIPTION-BASED NUCLEIC ACID
AMPLIFI CATION/DETECTION SYSTEMS
This is a continuing application under 35 U.S.C.

120/121 of USSN 07/064141 filed 19 June 1987, the contents of which are hereby incorporated by reference.

Field of the Invention
The present invention relates generally to advances in molecular biology and molecular genetics.

More particularly, the present invention relates to novel methods and kits containing requisite reagents and means for increasing the in vitro or x-vivo copy number of, i.e., amplifying, at least one selected segment (sequence) of nucleic acid or its complement or hybridizing homologous segment, in a sample comprising one or more nucleic acids, which may include RNA, DNA or both.

Among the applications in which the methods and kits of this invention find utility are 1) in analyses of body fluids and tissues for the detection of specific nucleic acid sequences characteristic of a genetic or pathogenic disease or condition by in vitro or x-vivo nucleic acid probe hybridization assays and 2) in the selective cloning of single-copy or rare or low-expression genes.

Background of the Invention
Much of the work in molecular biology, molecular genetics and applications thereof, such as for example, nucleic acid probe hybridization assays for blood-borne pathogens or defective genes, involves the detection or isolation of a particular nucleic acid sequence. A fundamental problem in such work is to detect or isolate and then quantitate a nucleic acid sequence of interest.

The problem has been a difficult one because biological materials, such as cell cultures, tissue specimens, and blood samples, typically are comprised of a complex mixtureof RNA and DNA entities of which at most only a minuscule fraction has a sequence of interest.

Indeed, practical applications of nucleic acid probe hybridization assays have been limited because the sensitivity of the assays, when carried out with reagents suitable for routine use and over acceptably short time periods, is too low to detect sequences of interest at the low concentration at which they occur in real samples.

Two fundamentally different approaches have been taken to address the problem of detecting a nucleic acid sequence of interest ("target segment") present at a low level in a complex mixture of nucleic acids.

In the first approach, the amount of nucleic acid (including the target segment) in a sample is not altered; instead, a signal-generating system is associated with the target segment and produces a detectable signal representative of the number of molecules of target segment. For example, a nucleic acid probe, with a sequence complementary to that of a subsegment of the target segment is linked to an enzyme such as alkaline phosphatase and is mixed with sample under hybridization conditions that will effect hybridization between the probe and the target segment (but not appreciably between probe and other nucleic acid sequences in the sample)
After removing enzyme-linked probe that failed to hybridize, a chromogenic substrate for alkaline phosphatase is added under suitable conditions and, in principle, a large number of detectable, colored molecules is rapidly produced for each probe molecule hybridized to target segment.

Numerous other systems for detecting nucleic acid sequences without altering the amount of target nucleic acid in the sample are known to the art. Another example is the commonplace labeling of a nucleic acid probe with radioactive atoms such as 32P and then detecting probe hybridized to target via amplified signal initiated by decay of the radioactive nuclei. Still another example involves linking to probe for the

target segment another nucleic acid that is capable of replication such that it can be readily detected by known techniques. Certain RNAs are known that are susceptible to (autocatalytically) replicase-induced replication by certain polymerase, such as bacteriophage RNA-dependent RNA polymerase, such asQp replicase and the replicase from brome mosaic virus (BMV).

In such a system, both an RNA and the RNA of complementary sequence are templates for replication by the RNA polymerase; consequently the amount of replicated RNA increases exponentially with time (as long as the number of RNA template molecules does not exceed the number of
RNA polymerase molecules in a system). See Miele petal.,
J. Mol. Biol. 171, 281 (1983). A system in which probe for a target segment is linked to an RNA capable of being replicated byQss replicase is described by Chu et al.,
Nucl. Acids Res. 14, 5591 (1986) and that by BMV replicase by Marsh et al., Positive Strand RNA Viruses, Alan R. Liss (publr.; New York)(1987; Proceedings of UCLA Symposium, 1986).

The first approach has two serious drawbacks.

First, in many instances, the copy number of target segment in a sample of practical size is so low that, even for reasonably rapid signal generating systems, the time required to generate detectable signal that is significantly above background is impracticably long.

Second, signal generation occurs at essentially the same rate from "background" signal generating molecules as from signal generating molecules associated with target. In any assay for a target segment, a signal due to "background" is unavoidable; that is, invariably there is some signal due to probe that non-specifically adheres to filters or other solid supports or hybridizes to segments with sequences closely similar to that of target segment. If the copy number of target is too low, the time constant ratio of signal from target plus background (i.e.

"signal") to signal from background (i.e. "noise") will be too low to be detectable significantly above background.

These and other drawbacks have led the art to a second approach of addressing the problem of detecting a target segment present at a low level in a complex mixture of nucleic acids.

This second approach is fundamentally different and involves increasing the copy number of the target segment itself, preferably to an extent greater than that of other sequences in a sample, particularly those that might erroneously be detected as target segment because of similarities in sequence. Examples of this second approach include various culture techniques in which cells that harbor the target segment are caused to increase in number, sometimes more rapidly than numbers of other cells, or in which particular nucleic acids (e.g., plasmids, RNAs) therein having disposed target segment are caused to increase in number.

Such culture techniques have the disadvantages of being cumbersome and problematic and time-consuming and manifest the inevitable: nucleic acids other than those which include target segment are simultaneously increased in copy number, thus potentially increasing "background."
Another disadvantage is the resultant growth of potentially dangerous organisms as a necessary step to achieve amplification.

Another example of this second approach is amplification of a DNA target segment in a so-called "polymerase chain reaction" (PCR"). This technique is a borrowed adaptation of known, naturally occurring processes occurring in the replicative process of, for example, single-stranded DNA genomes of certain virus entities, and in all events,.re reswents an application akin to cDNA preparationala Hong,Boscience Reports 1, 243 (1981); Cooke et al., J. Biol. Chem. 255, 6502 (1980); and Zoller et al., Methods inEnzymoloqy 100, 468-500 (1983).By this technique, a particular segment increases in copy number exponentially with a number of cycles, each of which entailsN byridizig to a 3'-terminal subsegment of each of the target segment and its complement (i.e., the segment of sequence complementary to that of target segment) aDMA primer, (2) extending each of the primers with a DNA polymerase, and (3) rendering single stranded by thermal denaturation the duplexes resulting from step (2). This technique is described in Saiki et al., Science2, 1350 (1985), andMullis et al., European Patent Application Publication Nos. 200362 and 201184. See also U.S. Patents 4683195 and 4683202.Reportedly, in applying the technique for 20 cycles over about 3 hours, the copy number of a target segment can be increased by a factor of about105.

Because only those segments to which a specific primer hybridizes with a sufficient stability to initiate chain extension by the polymerase to form the complement and which have a complement to which another specific primer hybridizes similarly to yield target segment upon chain extension, they increase exponentially in copy number while other non-target segments not erroneously hybridized with the employed primer increase, if at all, at most linearly in copy number as a function of number of cycles.

The polymerase chain reaction technique can greatly increase not only the copy number of target segment but also the ratio of the amount of target segment to that of background-causing segments in a sample.

Of course, it follows that this second approach can be applied to a sample in conjunction with use of the first approach applied with the amplified target segment to provide even a stronger detection signal.

It is an object of the present invention to solve the problems addressed by the prior art and to overcome the disadvantages enumerated in prior researchers endeavors to solve those problems. It is a further object of the present invention to provide a straightforward technique that can be utilized in an acceptably short time, employing the convenience of known reagents and having the precision necessary to reach consistent scientific results; one that can be employed in a reproducible assay setting and that is adaptable for use in kits for laboratory/clinical analyses.

It is thus an object of the present invention to increase the detectability of certain nucleic acid sequences (target segments) by amplification of said target sequences in an in vitro or x-vivo system devoid of the disadvantages enumerated thus far by prior art endeavors.

The present invention is concerned with a novel technique for carrying out the second approach to detecting a target segment present at a low level in a complex mixture of nucleic acids. It employs a novel RNA transcript production step in conjunction with, and derived from, a synthesized double-stranded cDNA copy of the target sequence as a complete cycle. Multiple cycles can be employed. By virtue of the transcription step being the dominant aspect of novelty, it is conveniently referred to herein as a transcription-based amplification system (TAS). The novel technique of the present TAS invention results in rapid increase in copy number of a selected target segment by making use of two properties of DNA-dependent RNA polymerase: (1) appreciable initiation of transcription from only a small number of sequences specific for each polymerase, see, e.g., Brown et al., Nucl.Acids Res. 14, 3521 (1986); and (2) rapid production of a large number of transcripts from each copy of a promoter (typically 102-104 per hour) recognized by an RNA polymerase. See Milligan et al., Nucleic Acids Res. 15, 8783 (1987). The technique of the invention can also utilize the ability of RNAs with certain sequences to be rapidly (autocatalytically) replicated by RNA-dependent RNA replicases. See also Miele et al., supra. In addition, it provides a standardization technique making possible unambiguous measurement of the amount of target DNA present in a sample.

The present invention (unless induced (autocatalytic) replication is employed) yields a single-stranded RNA transcript, or an RNA-DNA duplex formed therefrom when measures are not undertaken to prevent its formation, that has a subsegment that has the sequence of the target segment or the sequence complementary to that of the target segment, and that is present in large excess relative to nucleic acid with a subsegment of complementary sequence. This excess initially of a single-stranded RNA transcript is advantageous in certain methods for detecting amplified product with a labeled nucleic acid probe because little segment of complementary sequence is present to compete with probe for hybridizing to the amplified product.

Also, the single-stranded RNA transcript product hereof is struck-off more or less continuously and provides for direct detection of target segment without the necessity of cumbersome, error-prone repeated PCR cycles and strand separation. Such advantages are not provided by the PCR technique that yields double-stranded DNA (one strand of which comprises target segment and the other strand of which comprises complement of target segment) that need to be separated before detection and only after a large number of repeated cycles necessary to reach acceptable amplification levels.

The techniques of the present invention provide amplification of a selected target segment to an extent at least as great as the PCR technique over about the same period of time, but in a far more simplistic and reproducible manner and distinct from natural processes or other art

Summary of the Invention

We have discovered how bacteriophage DNAdependent RNA polymerase can be used to rapidly amplify (i.e., increase the copy number of) a selected target nucleic acid sequence (target sequence or segment) present in a sample of nucleic acids. Further, we have discovered how bacteriophage DNA-dependent RNA polymerase can be used together with bacteriophage RNA-dependent RNA polymerase to accomplish the same result. Heretofore, it has not been appreciated that such bacteriophage RNA polymerase could be used for this purpose.

The invention entails methods based on these discoveries and kits for carrying out the methods.

These methods and kits are particularly usefully applied in connection with nucleic acid probe hybridization assays for a nucleic acid which includes a target segment amplified in accordance with the invention.

Thus, the invention also entails methods, and kits for carrying out the methods, for detecting the presence or absence of a segment in a sample of a nucleic acid, which comprises a particular segment, by means of a probe hybridized to that segment after amplification in accordance with the invention.

The present invention is predicated on the novelty of certain RNA transcripts, their production, optional replication, and use, to achieve desired amplification and detection of corresponding (in sequence) target nucleic acid sequence. The invention is practiced in an in vitro or x-vivo setting, and is employed conjunctively with the synthesis of a double-stranded cDNA copy of the target sequence in order to produce a double-stranded nucleic acid template used in turn for production of said RNA transcripts. This process of double-stranded cDNA synthesis and RNA transcription constitutes a single cycle of the present transcription-based amplification system (TAS). If desired, this cycle may be repeated in order to achieve even higher levels of amplification.By virtue of the method by which they are produced (and reproduced), the transcripts correspond (identically or complementarily) in sequence to a target nucleic acid sequence contained in an original sample amongst a mixture of nucleic acids, and therefore, the presence of the transcripts in amplified form provides for their detection, and hence by correspondence, the in vitro or xvivo detection of the presence of the target nucleic acid sequence instaid sample.

Thus, the present invention involves the in vitro or x-vivo detection of at least one specific nucleic acid sequence (target sequence or segment) in a sample containing nucleic acid. The present invention reduces to a method comprising preparing a double-stranded nucleic acid containing a sequence corresponding to a target sequence operably linked to a promoter therefor, employing said double-stranded nucleic acid as a double-stranded nucleic acid template for the preparation of a plurality of RNA transcripts therefrom, each bearing an RNA sequence corresponding to said target sequence, and detecting the presence of said RNA sequence and by analogy the presence of target sequence.

The present invention is directed to all methods and means associated with the preparation and use of such
RNA transcripts. Thus, the present invention is directed to the optionally repetitive method of preparing said double-stranded nucleic acid template defined above comprising providing a first nucleic acid primer containing a promoter sequence operably linked to a sequence corresponding to a segment of a target sequence, as defined above, hybridizing under suitable conditions said first nucleic acid primer with target sequence in a sample containing nucleic acid, extending the hybridized said first nucleic acid primer in a polymerase extension reaction complementarily to the target sequence to form a corresponding duplex nucleic acid, separating the strands of said duplex, hybridizing to the separated promoter containing sequence strand under suitable conditions a second nucleic acid primer at the end opposite said promoter sequence, and extending the hybridized said second nucleic acid primer in a polymerase extension reaction complementarily to said promoter containing sequence.

The present invention is further directed to further, and alternative, methods and means of preparing said double-stranded nucleic acid template(supra), for example, an essentially single-pot reaction comprising providing a first nucleic acid primer containing a promoter sequence operably linked to a sequence complementary to a segment of a target sequence and a second nucleic acid primer having a sequence identical to a segment of a target sequence, said primers corresponding to different regions of said target sequence but not, or not substantially, overlapping in their correspondence to the target and being selected such that an extension product of one when separated from its complement can serve as a template for an extension product of the other, contacting a sample containing nucleic acid including target sequence with said primers under sequential hybridizing and strand separation conditions so as to produce, in turn, extension products of said primers.

The present invention is further directed to methods and means of employing said double-stranded nucleic acid supra., as a template for the preparation of a plurality of RNA transcripts therefrom in a reaction catalyzed by a DNA-dependentkNA polymerase that recognizes the promoter thereof, and detecting and measuring, the presence of said RNA transcripts.

The present invention isfurtner directed to methods and means to standardize the amountof target sequence detected (by correspondence to the amount of RNA transcript detected) by further correlationtc the presence of known nucleic acid used as an internal standard. The copy number of the standard is predetermined, the standard shall experience the same conditions, hence fate, during the practice of this invention as does target sequence and it shall therefore serve as an evaluation means in determining relative amounts of transcripts prepared in parallel for it and for target sequence.

The present invention is further directed to the further replication of obtained RNA transcripts defined above via the presence therein of replicase recognition sites. This is conveniently accomplished by providing said first nucleic acid primer defined above additionally bearing a replicase recognition site sequence, preferably between the promoter sequence and sequence corresponding to target sequence, and/or additionally containing a replicase recognition site on the second nucleic acid primer defined above. On subsequent transcription, the consequent transcripts bear the replicase recognition site(s), such that presence of a replicase (in the reaction locus or kit for example) (autocatalytically) induces replication of the transcripts producing additional copies to further facilitate detection.

The present invention is further directed to kits comprising requisite reagents and associated means useful in the in vitro or x-vivo detection of at least one specific nucleic acid sequence (target sequence) in a sample containing nucleic acid, employing the methods and means defined sura.

Detailed Description of the Invention
1. BriefDescrintion of the Drawings
Figures IA, 1B and 1C illustrate a method according to the invention for amplifying a target segment of a nucleic acid (Nucleic Acid A), which is a DNA or RNA, wherein many copies of a first RNA (RNA I) with a segment with a sequence complementary to that of target segment are made.

Figures 2A, 2B, 2C illustrate the further amplification according to the invention of a segment of an RNA I, made as illustrated in Figures IA, 1B and IC, to make many copies of a second RNA (RNA II) with a segment with a sequence the same as that of a subsegment of the target segment, which was amplified to make the RNA I.

Figure 3 depicts autoradiograms showing various concentrations of HIV RNA amplified by TAS simultaneously with a fixed concentration of human p-globin nucleic acid.

Figure 4 displays a general strategy whereby RNA produced through a DNA-dependent RNA polymerase yields an
RNA molecule that can serve as a template for an RNAdependent replicase.

2. General Methods and Definitions
Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques of the present invention such as: DNA probe or primer preparation, including DNA synthesis; hybridization methodology including variations in stringency conditions for producing more or less hybridization certainty depending upon the degree of homology of the primer to a target DNA sequence; identification, isolation or preparation of promoters, or more specifically promoters or sites recognized by bacteriophage DNA-dependent RNA polymerase and bacteriophage RNA-dependent RNA polymerase, or in the employment of eukaryotic systems, viral DNA- and RNAdependent RNA polymerases, for example, adenovirus encoded
RNA polymerase and brome mosaic virus RNA polymerase; conditions conducive to the production of RNA transcripts, including so-called transcription enhancer sequences; the mechanism and methodology for (induced) replication; polymerase chain reaction methods including the reagents used therein; and so forth. See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, (1982), and the various references cited therein; U.S. Patent 4683195; U.S.

Patent 4683202; Hong, Bioscience Retorts 1, 243 (1981);
Cooke et al., J. Biol. Chem. 255 6502 (1980); and Zoller et al., Methods inEnzvmoloav 100, 468-500 (1983); Crea etal., Nucleic Acids Res. 8, 2331 (1980); Narang etal.,

Meth.Enzvm. 68, 90 (1979); Beaucage et al., Tetrahedron
Letters 22, 1859 (1981); Brown et al., Meth. Enzvm. 68, 109 (1979); Caruthers et al., Meth. Enzym. 154, 287 (1985); Hitzeman et al., J. Biol. Chem. 255, 2073 (1980);
Lee et al., Science 239, 1288 (1988); Milligan et al.,
Nucleic Acids Res. 15, 8783 (1987); Miller et al.,Virology 125, 236 (1983), Ahlquist etal., J. Mol. Biol.

153, 23 (1981); Miller et al., Nature 313, 68 (1985);
Ahlquist et al., J. Mol. Biol. 172, 369 (1984); Ahlquist et al., Plant Mol. Biol. 3, 37 (1984); Ou et al., PNAS 79, 5235 (1982); Chu et al., Nucl. Acids Res. 14, 5591 (1986);
European Patent Application Publn. No. (EPA) 194809; Marsh et al., Positive Strand RNA Viruses, p. 327-336, Alan R.

Liss (publ.; New York) (1987; Proceedings of UCLA
Symposium, 1986); Miller et al., J. Mol. Biol. 187, 537 (1986); Stoflet et al., Science 239, 491 (1988); and Murakawa etal., DNA 7, 287 (1988).

All of the aforecited publications are by this reference hereby incorporated by reference herein.

By the term "promoter" is meant a nucleic acid sequence (naturally occurring or synthetically produced or a product of restriction digest) that is specifically recognized by an RNA polymerase that binds to a recognized sequence and initiates the process of transcription whereby an RNA transcript is produced. It may optionally contain nucleotide bases extending beyond the actual recognition site, thought to impart additional stability toward degradation processes. In principle, any promoter sequence may be employed for which there is a known and available polymerase that is capable of recognizing the initiation sequence. Typical, known and useful promoters are those that are recognized by certain bacteriophage polymerase such as bacteriophage T3, T7or-SP6. SeeSiebenlist'et al., Cell 20, 269 (1980).These are but examples of those polymerase which can be employed in the practice of the present invention in conjunction with their associated promoter sequences.

The "RNA transcript" hereof is the ribonucleic acid sequence produced after transcription initiation following RNA polymerase recognition of the promoter sequence (Seesura). The production of such transcripts is more or less continuous, dependent in part on the amount of polymerase present.

By the term "primer" in the present context is meant a nucleic acid sequence (naturally occurring or synthetically produced or a product of restriction digest) that has sufficient homology with the target sequence such that under suitable hybridization conditions it is capable of hybridizing, that is binding to, the target sequence.

A typical primer is at least about 10 nucleotides in length, and most preferably is of approximately 35 or more nucleotide bases in length, and in its most preferred embodiments, it shares identity or very high homology with the target sequence See, for example, EPA 128042 (publd.

12 Dec 84).

The term "operably linked" in particular in connection with the linkage of a promoter sequence within a primer sequence, refers to the functionality of the ultimate "double-stranded nucleic acid template" of the present invention such that it, the template, is capable of producing corresponding RNA transcripts when the promoter is recognized by the suitable polymerase--see sura.

The primer extension reaction to produce a duplex is known per se. See references suPra. Polymerase useful for this purpose include E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, reverse transcriptase, and so forth.

The techniques of forming a detection signal such as via radioactive labeling or chromogenic means using a chromogenic susceptible enzyme are also well known and documented in the art. See discussion sura.

The use of a "replicase" for (autocatalytic) induction of replication of the RNA transcripts of the present invention are generally known in the art.

Suitable examples of such replicases that are useful in the present invention include the so-calledQss virus replicase that recognizes certain nucleic acid sequence sites at both the 3'- and 5'- ends of the given

RNA transcript and the so-called brome mosaic virus (BMV) as well as the alpha virus replicases which are thought to recognize nucleic acid sequence sites at the3' end of a given RNA transcript. These replicases serve to replicate, that is reproduce, the RNA transcripts and complements so as to multiply copies thereof. When such enzyme is present in the reaction locus during the process of transcription, it can be foreseen that the multiple transcripts that are produced during transcription can themselves undergo replication so as to exponentially increase the amount of RNA transcript product.

Internal standardization is defined as a process which is used to: a) insure that the TAS amplification process has not failed because of a procedural error, and b) measure the levels of target nucleic acid relative to a predetermined quantity of nucleic acid which is always associated with the sample of interest. Such internal standardization occurs by coamplifying a portion of the target sequence, as well as an endogenous sequence, in the same reaction. For example, by knowing the cell count present within the biological sample, a single-copy gene(e;q., B-globin) could be used as an internal standard and, since it is not expressed in the form of RNA, its initial copy number is equal to two times the total number of cells in the sample.By coamplifying portions of thess-globin and target sequences of interest, the ratios of the amplified signals can be compared to quantitate the levels of target sequence of interest. Since every cell has two copies (in diploid cells) of this internal standard, irrespective of whether the biological sample contains separate target sequences(e.a., HIV), each sample is expected to produce a positive amplification signal resulting from the internal standard. See Groudine et al., Nucleic Acids Research 12, 1427 (1984) and
McKnight, Cell 31, 355 (1982). See also British Patent
Application Publn. No. 2187283 A (publd. 3Sept 87).

3. Detailed Description of Preferred Aspects
In one of its aspects, the invention is a method for amplifying a target nucleic acid segment of Formula I 3'-(first subsegment)t-(second subsegment)t (third subsegment)t-5'
I wherein (first subsegment)t is a nucleic acid segment of known sequence of at least 10 nucleotides adjoining the 3'-terminus of (secondsubsegment)t, (second subsegment)t is a nucleic acid segment of0 or more nucleotides, and (third subsegment)t is a nucleic acid segment of known sequence of at least 10 nucleotides adjoining the 5'-terminus of (secondsubsegment)t, which method comprises::
(1) hybridizing to (first subsegment)t of said target segment a first primer, which is a single-stranded DNA which comprises a 3'-terminal subsegment of Formula II 5'-(promoter)I-(variable subsegment)I (3'-primersubsegment)I-3'
II wherein (promoter)I is a single-stranded DNA segment with the sequence of the plus-strand of a bacteriophage
DNA-dependent RNA polymerase-specific promoter, (variable subsegment)I is a single-stranded DNA segment of0 to 100 nucleotides adjoining the 3'-terminal nucleotide of (promoter)I and the 5'-terminal nucleotide of (3'-primer subsegment)I, and (3'-primer subsegment)I is a single-stranded DNA segment of at least 10 nucleotides with a sequence which is complementary to the sequence of a subsegment of (first subsegment)t which terminates with the 3'-terminal nucleotide of (firstsubsegment)t, said (promoter)I adjoining the 5'-terminus of said (3'-primer subsegment)I, if said (variable subsegment)I has 0 nucleotides;;
(2) extending said first primer, hybridized in accordance with step (1), in a reaction catalyzed by a first DNA polymerase to make a first complementary DNA segment, which comprises a subsegment of Formula III 5'-(promoter)I-(variable subsegment)I (first subsegment) tc (second subsegment) tc (third subsegment)tc-3 ,
III wherein (first subsegment)tc is the DNA segment with the sequence complementary to that of (first subsegment)t, (second subsegment)ite is the DNA segment with the sequence complementary to that of (second subsegment)t, and (third subsegment)tc is the DNA segment with the sequence complementary to that of (third DNA polymerase is a reverse transcriptase;
(3) rendering single-stranded the duplex formed in the reaction of step <SEP> 10 <SEP> 20 <SEP> 30 <SEP> 40
<tb> <SEP> I <SEP> I <SEP> I <SEP>
<tb> <SEP> 5' <SEP> -AAGATCTATGTCCTAATTCAGCGTA <SEP> I <SEP>
ACAGCATATGTATGTTCAGGGA <SEP>
<tb> <SEP> 50
<tb> <SEP> ¦
<tb> AAGCTA-3' <SEP> (54 <SEP> bases), <SEP> as <SEP> a <SEP> core <SEP> sequence, <SEP> additionally <SEP> AU
<tb> rich sequence 5'-thereof may be used to enhance the replicase activity from BMV. This oligo is to be used as the second primer. The first primer used with this second primer is aT7-based, HIV-specific primer.

T7 promoter TCS = 87-34
EMI28.2

<tb> <SEP> +IS <SEP>
<tb> <SEP> 10 <SEP> 20 <SEP> 30 <SEP> 40 <SEP> 50
<tb>TAATACGACTCACTATA <SEP> GGGA <SEP> CACCTAGGGCTAACTATGTGTCCTAATAAGG
<SEP>
<tb> (52 bases).

Additional, non-limiting details to aid in understanding the invention are provided in the examples that follow:
4. Examples
EXAMPLE I
10ml of blood from a patient suspected of being infected with a human immunodeficiency virus-type 1 (HIV-1) is fractionated with a SepracellTM apparatus (Sepratech Corp., Oklahoma City, Oklahoma, U.S.A.) or, alternatively, with a Ficoll gradient, to isolate lymphocytes. The lymphocytes are then lysed and nucleic acid from them is isolated with an extractor apparatus (Molecular Biosystems, Inc., San Diego, California,
U.S.A.) or, alternatively, the lymphocytes are lysed by standard sodium dodecyl sulfate (SDS)-enzyme treatment and the nucleic acid isolated with reverse phase chromatography over DEAE cellulose. This is done as follows:
Spin down cells: 5k rpm for 4' from 1 ml Tris-buffered saline (TBS), pH 7.5.

Draw off supernatant.

Resuspend pellet in:
500Hl 0.3 M NaCl/20 mM Tris, pH 7.5 Mix well,
100pl 2% SDS then add
200p1 5 mg/ml proteinase K to
200pl 0.25 M EDTA pellet.

Vortex vigorously and incubate at 500C for 45', vortexing for 10-15 seconds every 10'.

Load onto drained extractor column and allow to enter.

Wash column lx 4 ml 0.3 M NaCl/20 mM Tris, pH 7.5.

Elute DNA/RNA with 4 ml 0.5 M NaCl/20 mM Tris pH 7.5.

To eluate add: 5M1 glycogen
400pl 3 M NaOAc
9.0 ml ice-cold EtOH
mix well
Precipitate at -200C overnight. A dry ice/ethanol bath for one hour may be used.

Spin down DNA/RNA for 15' at 10,000 rpm in a swinging bucket rotor; pour off EtOH and drain dry on a kimwipe ,
Lyophilize for 10'.

Resuspend pellet in 1701 TE.

Incubate at 370C for 5'.

Pour a 1 ml spin column as follows:
Plug a 1 ml syringe with a small amount of glass wool (autoclaved).

Fill syringe with TE (tris-EDTA) treated with diethyl pyrocarbonate (DEPC).

Quickly add sephadex G-50 fine (in TE; autoclaved).

Continue to add until the solid matrix mounds over the top of the syringe.

Spin 30 seconds at 1000 rpm in an IEC table-top centrifuge.

Wash with 100z1 TE, spin for 1' at medium speed (ca. 1000 rpm). Discard wash.

Load the extract onto the column. Spin again for 1'.

Rinse with 1501 TE, spin at 1000 rpm for 1'. Pool rinse and the first fraction. Samples may be split for multiple reactions at this step.

Add 1/10 volume 8 MLick. Mix with 2.5 x vol. 100% EtOH.

Vortex well. ppt. dry ice/EtOH for 30'.

Spin down for 15' top speed in a microfuge at 40C.

Dry pellet.

After the isolation, total nucleic acid from the sample is dissolved in10owl of TE buffer(lOmM Tris.Cl, lmM EDTA, pH8).10S1 of the100A1 of total nucleic acid is dissolved to a final volume of100 1 containing:
40mM Tris.Cl, pH8
25mM NaCl lOmM MgC12
10mM dithiolthreitol (DTT)
2mM spermidine
100zg/ml bovine serum albumin
40OmM each of dATP, dCTP, dGTP and TTP
30nM of the 101 base single-stranded DNA of sequence 5'-GAACGCGGCT ACAATTAATA CATAACCTTA TGTATCATAC
ACATACGATT TAGGTGACAC TATA GAATAC TTTCGTAACA CTAGGCAAAG
GTGGCTTTAT C -3' primer A, is added. 30nM of the 29 base
DNA of sequence 5'-ACACCATATG TATGTTTCAG GGAAAGCTA-3', primer B, is added.Primer B, which is the second primer, has the sequence of bases 5151-5179 in the SOR gene of the HIV-1. An alternative segment for the second primer, corresponding to bases 5357-5387 of the SOR gene of the
HIV-l, has the sequence 5'-GCACACAAGT AGACCCTGAA
CTAGCAGACC A-3', and, if used, is also present at 30 nM.

Primer A is the first primer; its 64 5'-nucleotides form the plus-strand of a promoter for the SP6 DNA-dependent
RNA polymerase. Its segment of sequence 5'-GAATAC-3' is (alternative subsegment)l, which includes the transcription start site (the 5'-G) and other bases apparently favored by the SP6 RNA polymerase at the 5'-end of sequences transcribed by it. Finally, the 31 3'-terminal nucleotides form (primer subsegment)l and have a sequence complementary to that of bases 5577-5547 in theSOR gene of an HIV-1 isolate (See Ratner et al., Nature 313, 277 (1985) for complete sequence.) (The HIV isolate is referred to in these Examples as "the HIV-1")).

Note that all oligonucleotides employed in these examples are made by solid-phase synthesis on an Applied
Biosystems, Inc. (Foster City, California, U.S.A.) automated synthesizer (Model No. 380A) and are purified chromatographically essentially to homogeneity by HPLC using a C8 reverse phase column. Alternatively, other commercially available synthesizers and standard purification procedures could be used to prepare the oligonucleotides.

The100z1 of solution is heated at 650C for 2 minutes and is then cooled to 420C over the course of 1 minute. This heating and then holding at 420C or above, in combination with the composition of the

solution, provides conditions of stringency sufficient to provide hybridization of (3'-primer subsequent)l, with sufficient stability to prime DNA synthesis, of high specificity to the sequence complementary to that of (3'-primer subsequent)l.

Then 10 units of avian myoblastosis virus (AMV) reverse transcriptase or 500 units of recombinant Moloney murine leukemia virus (MMLV) reverse transcriptase, as purchased from Life Sciences, Inc., St. Petersburg,
Florida, U.S.A., are added to the solution and the solution is incubated for 10 minutes at 420C. (One unit incorporates lnmole of TTP into acid-precipitable form in 10 minutes at 370C usingpoly(A).oligo(T)12,18 as template-primer as defined by Houts et al., J. Virol. 29, 517 (1979)). After the 10 minute incubation, the solution is placed in a boiling water bath for 1 minute. This heating causes strand-separation of the duplex formed by the reverse transcriptase.

Then the solution is cooled to 420C over 1 minute.

During this cooling, the second primer hybridizes to the 3'-terminal segment of the complement of target segment formed in the reaction catalyzed by the reverse transcriptase. Again, the hybridization conditions are sufficiently stringent for sufficiently specific hybridization between the second primer and sequence complementary to that of second primer.

After the cooling, 10 additional units of AMV reverse transcriptase or 500 additional units of cloned MMLV reverse transcriptase are added and further incubation for 10 minutes at 420C is carried out.

Then RNasinR brand ribonuclease inhibitor from
Promega Biotec, Madison, Wisconsin, U.S.A. is added (optionally) to a concentration of 1 unit per ml. (1 unit is the amount of inhibitor required to inhibit by 50% the activity of 5ng of ribonuclease A. Roth, Meth. Cancer
Res. 3, 151 (1976)). Further, each of the ribonucleoside 5'-triphosphates, ATP, GTP, CTP and UTP, is added to 400mM.Finally between 10 and 20 units of SP6
DNA-dependent RNA polymerase, purchased from Promega
Biotec, are added and the resulting solution is incubated at 420C for 30 to 60 minutes. (1 unit is the amount of the RNA polymerase required to catalyze the incorporation of 1 nmole of ribonucleoside triphosphate into acid insoluble product in 60 minutes at 370C under standard reaction conditions (40mM Tris.Cl, pH 7.9, 6mMMgC12, 10mM
DTT, 2mM spermidine, 0.5 mM of each of ATP, GTP, CTP and
UTP, 0.5Ci of 3H-CTP, lg of SP6 DNA and the enzyme in a total volume of50p1.))
Then each of the following oligonucleotides is added to bring its concentration to 30nM:
third primer: 5'-GAACGCGGCT ACAATTAATA CATAACCTTA TGTATCATAC ACATACGATT TAGGTGACAC TATA GAATAC ACTAATTCAT CTGTATTACT TTGACTGTTT
TTC-3'
fourth primer: 5'-TTTTTTGGTG TTATTAATGCTGCTAGTGCC-3,
In the third primer, as in the first, the 5'-terminal 64 bases are the promoter segment and the next 6 bases are the variable segment. The variable segment is the first six bases transcribed from the promoter by the SP6 RNA polymerase, and these bases are selected to enhance the level of such transcription. Finally, the(3'-primer subsegment)3 portion of the third primer is the 3'-terminal 33 bases, in the same sequence as bases 5388-5420 in the short open reading frame (SOR) gene of the HIV-1. The fourth primer has the sequence complementary to that of bases 5546-5517 of the SOR gene of the HIV-1.

After addition of the third and fourth primers, the solution is incubated at 420C for 1 minute. During this period, hybridization occurs between (3'-primer subsegment)3 of third primer and the first RNA formed in the reaction catalyzed by the SP6 RNA polymerase. Because of the stringency of the hybridization conditions, the (3'-primer subsegment)3 hybridizes, with stability sufficient for priming of DNA synthesis, with high specificity to the segment of complementary sequence in the first RNA.

After the incubation, 10 units of AMV reverse transcriptase or 500 units of cloned MMLV reverse transcriptase are added and the solution incubated for 10 minutes at 420C to form the third complementary DNA.

The solution is then suspended in a boiling water bath for 1 minute and cooled to420c over 1 minute to, first, render single-stranded the duplex between third complementary DNA and first RNA and, second, allow hybridization between fourth primer and third complementary DNA. As with the other hybridizations,

the conditions are sufficiently stringent that hybridization of fourth primer, with stability sufficient to prime DNA synthesis, occurs with high specificity to the segment of third complementary DNA of sequence complementary to that of fourth primer.

Then, again, 10 units of AMV reverse transcriptase or 500 units of cloned MMLV reverse transcriptase are added and the solution is incubated for 10 minutes at 420C.

Then RNasinR brand ribonuclease inhibitor is added (optionally) to 1 unit per ml, followed by 10-20 units of
SP6 RNA polymerase, and the solution is incubated for 30 minutes to 1 hour at 420C.

The resulting second RNA can then be detected by a nucleic acid probe hybridization technique.

EXAMPLE II
The procedure of Example I is followed, with a modification noted below, with three samples: (A) one of 10ml of human blood known to be free of HIV, (B) one of10ml of culture known to have about 103 HIV-I infected cells per ml, and (C) one of 10ml of blood from a person suspected of being infected with an HIV-1. The modification of the procedure is that analpha-32P-labeled ribonucleoside triphosphate is included as a substrate in the reaction catalyzed by the SP6 RNA polymerase to make the second RNA. The second RNA is, consequently,32P-labeled.

Sephacryl-S500TM macroporous beads are derivatized with a carboxyl-group-terminated linker (of formula-C(=NH)NH(CH2)5 CO2-) and then with 5'-(6-aminohexyl phosphoramidate)-derivatized oligonucleotide of sequence 5'-TGGTCTGCTAGTTCAGGGTC
TACTTGTGTG C-3' which is the sequence complementary to that of bases 5357-5387 in the SOR gene of the HIV-1.

(The sequence of bases 4901-4932 of the SOR gene occurs in any second RNA produced during amplification of nucleic acid from a sample.) Preparation of the beads was according to procedures described in commonly assigned
United States Patent Application Serial No. 895,756, filed
August 11, 1986, and incorporated herein by reference.

Briefly, the support materials are porous silicate glass or macroporous cross-linked dextran, derivatized with an amino-terminated linker, with substantially all of the amino groups that are not covalently joined through a phosphoramidate group to the terminal nucleoside of the capture probe being blocked from interacting nonspecifically with nucleic acid having an aliphatic acyl group; macroporous cross-linked dextran activated with cyanogen bromide, which reacts with amines to link to aminoalkylphosphoramidate derivatized oligonucleotides; and porous silicate glass, macroporous cross-linked dextran or divinylbenzene-crosslinked polystyrene derivatized with acarboxyl-terminated or succinimideester-terminated linker, with a fraction of the carboxyl groups joined in an amide linkage to one amino group of a diaminoalkane, the other amino of which is part of a phosphoramidate which, in turn, is bonded directly to the terminal nucleoside of the capture probe.Preferred support materials are long chain alkylamine-derivatized and carboxyl-derivatized controlled pore glass as sold by
Pierce Chemical Co., Rockford, Illinois, USA, under product numbers 24875 and 23735, respectively, in the form of beads with nominal pore diameter of 500 Angstroms and particle diameters between about 125 and 177 microns, and
SephacrylS-500, sold by Pharmacia Inc., Piscataway, New
Jersey, USA under Code No. 17-0475-01 in the form of beads with a wet diameter of about 40 to about 105 microns and an exclusion volume for dextrans of molecular weight above about 2x107 daltons, said Sephacryl to be derivatized with cyanogen bromide or with an amino-terminated or carboxylterminated linker. In one aspect the solid support is one of:
(A) porous silicate glass derivatized at silicons with
(1) groups of formula
-(CH2)n(NH)(Co)(CH2).CH3 and -(CH2)n(NH)(PO2y0-(Oligo), with substantially no silicon derivatized with a group terminated with an amino group, wherein c is0 to 5, n is 2 to 8, -0-(Oligo) is the oligonucleotide probe, and the oxygen atom bonded to (Oligo) is the oligonucleotide probe, and the oxygen atom bonded to (Oligo) is the 5' oxygen of the 5'-nucleoside or the 3'-oxygen of the 3'nucleoside of the probe; or
(2) groups of formula
-(CH2)n(NH)(CO)(CH2)mC02H and ~CH2)n(NH)(co)(cH2)m(co)(NH)(cH2)pNH(po2)o-(oligo) wherein m, n, and p are the same or different and are each 2 to 8; or

(B) a cross-linked dextran macroporous material derivatized at hydroxyl oxygens, which are on carbons of the sugar moieties which have at least one neighboring carbon with an underivatized hydroxyl, with
(1) groups of formula
-C(=NH)NH(CH2)q(NH)(Co)(CH2)CCH3 and -C(=NH)NH(CH2)p(NH)(P02)0-01igo), with substantially no hydroxyl oxygen derivatized with a group terminated with an amino group, or
(2) groups of formula
-C(=NH)NH(CH2)qCO2H and
C(=NH)NH(CH2)q(CO)(NH)(CH2)pNH(PO2)O-(Oligo), wherein c, q, and p are the same or different and q is 2 to 10; and
(C) divinylbenzene-crosslinked polystyrene derivatized at phenyl groups with groups of formula
-(CH2)sCO2H and
-CH2)s(CO)(NH)(CH2)pNH(PO2)O-(Oligo), wherein s and p are the same or different and s is 2 to 10. See Ghosh et al., Nucleic Acids Research 15, 5353 (1987).

Each of three batches of 50mg of Sephacryl beads, derivatized as described above, is soaked for 15 minutes at 370C C in250p1 of a prehybridization solution containing 0.1% SDS, 10% dextran sulfate, lmg/ml salmon sperm DNA, and 5x SSPE (0.75M NaCl, 50mM NaH2PO4, pH 7.4, and 5mM EDTA). After the soaking, the bead material is pelleted by centrifugation and prehybridization solution is removed by aspiration.

The nucleic acid from the amplification procedure on each of the three samples is isolated by ethanol precipitation and is then dissolved to250H1 in solution that is the same as pre-hybridization solution but lacks the salmon sperm DNA. Each of the resulting nucleic acid solutions is then combined with one batch of prehybridized oligonucleotide-derivatized Sephacryl beads and the resulting mixture is incubated with gentle agitation at 420C C for 90 minutes.

The Sephacryl beads are then pelleted by centrifugation, hybridization solution is removed by aspiration, and the beads are then washed three times, 10 minutes each at 370C C, in lml of a solution of 2 x SSC (0.30M NaCl, 0.03M Na.Citrate, pH 7.0). After each wash, the beads are pelleted by centrifugation and solution is removed by aspiration.

Immediately after the third wash, the beads are subjected to Cerenkov counting.

The beads with amplified nucleic acid from sample A produce a low level of counts, barely above background counts from scintillation fluid alone. The beads with amplified nucleic acid from sample B produce counts at a much higher level than that observed with beads associated with sample A. The beads with amplified nucleic acid from sample C, if they produce a level of counts significantly above the level produced with beads associated with sample
A, indicate that the person, whose blood was taken for sample C, is infected with HIV-1.

EXAMPLE III
The procedures of Examples I and II are carried out with T7 RNA polymerase (Promega Biotec) in place of SP6RNA olymerase, with each of the subsegment5'-(promoterl-(variable subsegment)l-3' of first primer and the subsegment5'-(promoter)3-(variable subsegment)3-3' of third primer having the sequence 5'-TAATACGACT CACTATA GGGA-3', wherein the 17 5'-terminal bases are the promoter subsegment and the 4 3'-bases are the variable subsegment. The variable segment corresponds to the 4 5'-terminal bases of the transcript from the promoter. Ribonuclease inhibitor is not used in any step in this process, at least with the polymerase preparation from Promega Biotec.

EXAMPLE IV
The procedures of Examples I and II are carried out with T3 RNA polymerase (from Stratagene, San Diego,
California) in place of the SP6 RNA polymerase and with the following segment used as the (promoter)l-(variable subsegment)l subsegment of first primer and the(promoter)3-(variable (variable subsegment)3 subsegment of third primer: 5'-TATTAACCCT CACTAAA GGGA-3', where the 17 5'-terminal bases are the promoter segment and the 4 3'-terminal bases the variable segment, including the 5'-terminal 4 bases in transcripts from the promoter.

EXAMPLE V
Employing the T7 RNA polymerase as in Example III, target segment in the genome of HIV from human blood samples is amplified using the following primers in place of the primers specified in Example I:

First Primer: 5'-TAATACGACT CACTATA GGGA
TCTAATTACT ACCTCTTCTTCTGCTAGACT-3' wherein the 30 3'-terminal bases are complementary in sequence to bases 7076-7047 of the ENV gene of HIV-1.

Second Primer: 5'-ACAAGTTGTA ACACCTCAGT
CATTACACAG-3' with the sequence of bases 6838-6866 in the
ENV gene of HIV-1.

Third Primer: Same 21 5'-terminal bases as first primer of this Example adjoined to the following 27 3'-terminal bases: 5'-AAAGGTATCC TTTGAGCCAA TTCCCATA-3', which have the sequence of bases 6875-6902 in the ENV gene of the HIV-1.

Fourth Primer:5'-AGTTGATACTACTGGCCTAATT-3', with the sequence complementary to that of bases 7033-7007 in the ENV gene of HIV-1.

EXAMPLE VI
Employing the T7 RNA polymerase as in Example III, target segment in the genome of HIV from human blood samples is amplified using the following primers in place of the primers specified in Example I:
First Primer: Same 21 5'-terminal nucleotides as the first and third primers of Example V adjoined to the following 31 3'-terminal nucleotides: 5'-CACCTAGGGC
TAACTATGTG TCCTAATAAG G-3', which have the sequence complementary to that of bases 5471-5441 of the SOR gene of HIV-1.

Second Primer: 5'-ACACCATATG TATGTTTCAG
GGAAAGCTA-3', which has the same sequence as bases 5151-5179 of the SOR gene of HIV-1.

Third Primer: Same 21 5'-terminal nucleotides as the first and third primers of Example V adjoined to the following 31 3'-terminal nucleotides: 5'-AAGAATAAGTTCAGAAGTACACATCCCACT-3', which have the same sequence as bases 5220-5249 of the SOR gene of the HIV.

Fourth Primer:5'-TGGTCTGCTAGTTCAGGGTCTACTTGTGTC- 3', which has the sequence complementary to that of bases 5387-5357 in the SOR gene of HIV-1.

EXAMPLE VII
The procedure of Example I is followed for the isolation of nucleic acid from: 1) one sample containing 103 HIV-infected CEM cells mixed with106 uninfected CEM cells (Cancer Center Research Foundation; CCRF-CEM; ATCC
No. CCL 119); and 2) one sample containing 106 uninfected
CEM cells. These samples are resuspended in 100pl 10 mM
Tris-HCl, pH 7.4, 1 mM EDTA (TE) after the ethanol precipitation step to concentrate the sample obtained from the ExtractorTM column (MBI). The resuspended samples are fractionated on a Sephadex G-50 fine spin column (Maniatis sura) eluting with TE. The eluted samples are concentrated by ethanol precipitation (in 0.8 M LiCl, 3 volumes ethanol, in dry ice/ethanol bath for 15 minutes).

The precipitated sample is pelleted by centrifugation.

The pellet is drained, dried, and then resuspended in 100pl containing 40 mM Tris-HCl, pH 8.1, 8 mM MgCl2, 25 mM
NaCl, 2 mM spermidine, 5 mM dithiothreitol, 100pM each dATP, dGTP, dCTP, and dTTP, 1 mM each rATP, rCTP, rGTP, and rUTP, 100jig/ml BSA (nuclease-free) and 250 nM each of
DNA oligonucleotide primer A (5'-
AATTTAATACGACTCACTATAGGGACACCTAGGGCTAACTATGTCCTAA
TAAGG-3) and primer B(5'-ACACCATATGTATGTTTCAGGGGAAAGCTA- 3').

As controls, duplicate samples of purified HIV RNA at a concentration of 0.01 fm were resuspended in 100jil of the buffer described above. Finally, a 10-fmp-globin sequence contained in plasmidHB19A [Wallace et al., Nucl.

Acids Res.9 3647 (1981), which had been linearized by
HindIII, was resuspended in 100jil of the buffer described above, except without the oligonucleotide primers.

Oligonucleotide primer D (5'-ACATTGCTTCTGACACAACTGTGTTCAand primer C (5'-TAATACGACTCACTATAGGGACAAAGGACTCAAA-3'), which are specific forB-globin sequence, were added to thep-globin reaction at 250 nM each.

Except for theB-globin sample, the reactions are heated to 650C for 1'. TheB-globin sample is boiled for 1'. All samples are cooled to 420C for 1 minute; then, 10 units of AMV reverse transcriptase are added. The reactions are incubated for 15 minutes at 420C, boiled for 1 minute, and then cooled at 420C for 1 minute. Ten additional units of AMV reverse transcriptase are added, incubating at 420C for 15 minutes. One hundred units of
T7 RNA polymerase are added to the reactions, and the reactions are incubated at 370C for 30 minutes.

The samples are boiled for 1 minute and cooled at420C for 1 minute, followed by the addition of 10 units AMV reverse transcriptase. The reactions are incubated at 420C for 15 minutes, boiled for 1 minute and cooled to 420C for 1 minute. An additional 10 units of reverse transcriptase are added, followed by incubation at 420C for 15 minutes. One hundred units of T7 RNA polymerase are added, with a 30-minute incubation at 370C. This cycle is repeated two additional times.

The amplified target is then detected using
OligoBeadsTM as described below.

Sephacryl beads containing HIV-I-specific oligonucleotides were prepared as described and stored in TE at 40C at a concentration such that250jim of the suspension contains 50mg of Sephacryl beads.

Oligonucleotides were synthesized and HPLC purified as described.

The oligonucleotides employed for both attachment to the beads and detection are homologous to the SOR region of the HIV-1 genome. The oligonucleotides used in these studies were 86-31 (detection oligo nucleotide) (5'
GCACACAAGTAGACCCTGAACTAGCAGACCA-3') and 87-83 (capture oligo nucleotide on the bead: 5'-ATGCTAGATTGGTAATAACAACATATT-3'), which are homologous to the nonsense strand of the SOR region, and separated by approximately 100 nucleotides. For detection, the oligonucleotides were end labeled with32-P according to a standard protocol. The unincorporated label was removed by gel filtration on a Sephadex G-50 fine column, and the oligonucleotide was stored at-200C.

In a typical bead based sandwich hybridization system (BBSHS) experiment, the target and 32P-labeled detection oligonucleotide are denatured in10U1 of TE containing 0.2% SDS at 650C for 5 minutes in an Eppendorf tube. To this,10p1 of 2X Solution Hybridization Mix (10X
SSPE/10% Dextran Sulfate) are added. The solution is mixed, centrifuged 2 seconds, and incubated at 420C for 1 hour.

During this time the Sephacryl beads are prehybridized. The stock suspension of beads is mixed well, and250A1 aliquots (50 mg of beads) are transferred to
Eppendorf tubes with mixing between each removal to ensure a uniform suspension. The aliquots are centrifuged for 10 seconds, and the TE is removed with a drawn-out Pasteur pipet. After250p1 of Hybridization Solution (5X SSPE [0.9M NaCl, 50 mM NaH2PO4,pH 7.4, lmM EDTA] 10% DextranSulfate/0.1% SDS) is added, the beads are suspended by gentle shaking and incubated 370C for 30-60 minutes with occasional mixing.Immediately prior to the capture step, the beads are centrifuged for 10 seconds, the prehybridization solution is removed,80p1 of
Hybridization Solution at 370C are added, and the beads are returned to 370C.

The solution hybridization is centrifuged for 2 seconds and transferred to the beads and Hybridization Solution. The beads are suspended and incubated at 370C for 1 hour with frequent mixing to maintain the suspension.

Following the capture, the beads are centrifuged 10 seconds and the Hybridization Solution, containing uncaptured target and detection oligonucleotide, is transferred to a scintillation counter vial. The beads are then washed 5 times with 2X SSC at 370C. The first 3 washes are rapid; 1 ml of wash is added, the beads are mixed well and centrifuged 10 seconds, and the wash is transferred to a counting vial. For the final 2 washes,l ml of wash is added and the beads are mixed and incubated at 370C for 5 minutes before being centrifuged. Each wash is counted separately to monitor the procedure.

Cerenkov counts of the Hybridization Solution, 5 washes, and beads are measured for 5-10 minutes. Counter background is subtracted from all samples. The fm target detected is calculated as follows:
(CPM on Beads/Total CPM) X fm Oligonucleotide
Where total CPM is the sum of the CPM for the
Hybridization Solution, 5 washes, and beads.

DETECTION OF AMPLIFIED TARGETS BY BBSHS
TARGETyl USED (FM) DETECTED
10-15 MOLES 103 HIV infected cells; 0.33 0.06 106 CEM uninfected cells 0.66 0.13 106 uninfected CEM cell 0.070.0l
0.14 0.015
0.7 0.01
10 0.008
0.01 fm HIV RNA 0.007 0.14
0.014 0.29 10 fmp-globin DNA 10 0.014
EXAMPLE VIII
The procedure of Example I is followed for isolation of nucleic acids from two samples: a) 103 HIVinfected CEM cells with 106 uninfected CEM cells; and b) 106 uninfected cultured CEM cells to which 0.01 fmoles of purified HIV are added after extraction. The procedure is then modified by further purification through a Sephadex
G-50 (fine) spin column (Maniatis suPra) eluting with TE after the Extractor column step.This is followed by an ethanol precipitation of the nucleic acid (in 0.8 M LiCl and 2.5 volume of EtOH). The nucleic acid is then resuspended in 100jil containing:
40 mM Tris-HCl, pH 8.1
8 mMMgC12
25 mM NaCl
2 mM spermidine
5 mM DTT (dithiothreitol) 100HM dATP, dTTP, dCTP, dGTP (each)
1 mM rATP, rCTP, rGTP, rUTP 100jig/ml BSA nuclease-free 250 nM 29 bp DNA oligonucleotide with the sequence
5 'ACACCATATGTATGTTTCAGGGAAAGCTA-3' (Primer B) 250 nM 56 bp DNA oligonucleotide with the sequence
5,-
AATTTAATACGACTCACTATAGGGACACCTAGGGCTAACTATGTGTCCTAA
TAAGG-3') (Primer A)
The 100jil of solution are heated to 650C for 1 minute and cooled to 420C over the course of 1 minute.

This heating and then holding at 420C or above, in combination with the composition of the solution, provides conditions of stringency sufficient for hybridization of (3'-primer subsegment)I, (see Figure 1) with sufficient stability to the sequence complementary to that of (3'-primer subsegment)I, in the target HIV RNA with high specificity.

Then, 10 units of avian myoblastosis virus (AMV) reverse transcriptase (Life Sciences, Inc.) are added to the solution, and the solution is incubated for 10 minutes at 420C. [One unit incorporates 1 nmole of TTP into acidprecipitable form in 10 minutes at 370C using poly(A)oligo (T)12-18 as template-primer, as defined by Houts et al. J. Virol. 29, 517,(l979). After a 10 minute incubation, the solution is placed in a boiling water bath for one minute. This heating causes strand separation of the duplex formed by reverse transcriptase and inactivation of the reverse transcriptase.

Then, the solution is cooled to 420C over one minute. During this cooling, the second primer, primer B, hybridizes to the 3'-terminal end (thirdsubsegment)t2c, see Figure 1, of the target complement of DNA strand formed in the reaction catalyzed by the reverse transcriptase in the previous step. Again, the hybridization conditions are sufficiently stringent for specific hybridization between the second primer and the sequence complementary to that of the second primer.

After cooling, 10 additional units of AMV reverse transcriptase are added and further incubation is carried out for 10 minutes at 420C.

One hundred units of T7 polymerase are added to the reaction. The reaction is then incubated at 370C for 30 minutes. An RNA transcript is synthesized which is complementary to the original target HIV RNA

starting from the T7 promoter present at the 5' end of the duplex
DNA template newly synthesized by the reverse transcriptase. The transcript has the sequence 5'-GGGA, then continues with sequence which is complementary to the second subsegment of the target sequence (i.e., secondsubsegmenttcr, see Figure 1). Since the reverse transcriptase has not been inactivated prior to this step, and since primer B is present, as well as the deoxynucleotide triphosphates, a secondary synthesis occurs at this stage.Primer B hybridizes to the 3' region of the newly synthesized RNA transcript (thirdsubsegment)t2crt see Figure 1, which is complementary to B primer. The reverse transcriptase (which had been added at the previous step) synthesizes a DNA strand using the newly synthesized RNA transcript (made by T7 polymerase) as a template, and using oligonucleotide B as the primer at the 5' end.

The reaction is then boiled for one minute to denature the RNA:DNA duplex. The reaction is then cooled to 420C over one minute. During this cooling step, the target complementary segment of primer A hybridizes to the
DNA strand synthesized during the previous step. At the same time, primer B hybridizes to its complementary sequence on the RNA transcript synthesized in the previous step.

After cooling, 10 additional units of AMV reverse transcriptase are added, and further incubation for 10 minutes at 420C is carried out. Reverse transcriptase synthesizes DNA complementary to the HIV targetmusing oligonucleotide A as a primer at the 5' end and DNA made in the previous step as a template. A second DNA strand is synthesized using oligonucleotide B as a primer and the RNA transcript made in the previous step as a template.

The reaction is boiled for one minute to denature the DNA duplex and the RNA:DNA duplex. The reaction is cooled to 420C over one minute. Primer A hybridizes to the cDNA made using the RNA transcript as template in the previous step. (If the 3' end of the template strand is used as a reverse transcriptase primer also, a product identical to the end product of the next reaction is produced.) Primer B hybridizes to the DNA strand which is complementary to the target HIV RNA. This second synthesis produces a product which is a duplex DNA containing a T7 promoter sequence at its 5' end, as well as a 4 bp "variable" segment, 5'-GGGA-3'.

One hundred units of T7 polymerase are added to the reaction. The reaction is incubated for 30 minutes at370 C. The T7 RNA polymerase uses the double-stranded duplex DNA containing a duplex T7 promoter as a template to transcribe an RNA which is complementary to the target second subsegment containing the additional sequence 5'
GGGA-3' at its 5' end.

This cycle (boil 1', 1' at 420C, RT 10' at 420C, RT10'art 420C, T7 polymerase 370C 30') can be repeated as many times as needed to achieve the desired amplification of target sequence. The resulting product can then be detected by a nucleic acid probe hybridization technique.

EXAMPLE IX
Purified HIV RNA at a concentration of 1 fmole was resuspended in:
40 mM Tris-HCl, pH 8.1
8 mM MgCl2
25 mM NaCl
2 mM spermidine
5 mM dithiothreitol
100pM dATP, dTTP, dCTP, dGTP (each)
1 mM each rATP, rCTP, rGTP, rUTP
100pg/ml BSA nuclease-free
250 nM 29 bp DNA oligonucleotide with the sequence
5' -ACACCATATGTATGTTTCAGGGAAAGCTA-3' (primer B)
250 nM 56 bp DNA oligonucleotide with the sequence 5 -
'AATTTAATACGACTCACTATAGGGACACCTAGGGCTAACTA
TGTGTCCTAATAAGG-3' (primer A)
The 100jil of solution are heated to 650C for 1 minute and cooled to 420C over the course of 1 minute.

This heating and cooling step, in combination with the composition of the solution, provides conditions of stringency sufficient for the hybridization of primer A with sufficient stability to the sequence complementary to that of the primer A region in the target HIV RNA [(firstsubsegment)t2, in Figure 1] with

high specificity.

Then, 10 units of avian myoblastosis virus (AMV) reverse transcriptase (Life Science, Inc.) are added to the solution, and the solution is incubated for 10 minutes at 420C. After a ten-minute incubation, the solution is placed in a boiling water bath for one minute. This heating causes strand separation of the duplex formed by reverse transcriptase and inactivation of the reverse transcriptase.

The solution is cooled to 420C over one minute.

During this cooling, the second primer B hybridizes to the 3' end of the newly synthesized target-complementary strand [or (thirdsubsegment)t2e in Figure 1]. Again, the hybridization conditions are sufficiently stringent for specific hybridization between the second primer and the sequence complementary to that of the second primer.

After cooling, 10 additional units of AMV reverse transcriptase are added and further incubation is carried out for ten minutes at 420C.

One hundred units of T7 RNA polymerase (New England
Biolabs) are added to the reaction. The reaction is then incubated at 370C for 30 minutes. An RNA transcript is synthesized which is complementary to the original target
HIV RNA starting from the T7 promoter present at the 5' end of the duplex DNA template synthesized by the reverse transcriptase. The RNA transcript has the sequence 5'
GGGA, then continues with the sequence which is complementary to the second subsegment of the target sequence (i.e., secondsubsegmenttcr, Figure 1). Since the reverse transcriptase has not been inactivated prior to this step, and since primer B is present as well as the deoxynucleotide triphosphates, a secondary synthesis occurs at this stage. Primer B hybridizes to the 3' region of the newly synthesized RNA transcript [(third subsegment)t2crl r, see Figure 1] which is complementary to primer B. The reverse transcriptase (which had been added at the previous step) synthesizes a DNA strand using the newly synthesized RNA transcript (made by the T7 RNA polymerase) as a template, and oligonucleotide B as a primer at the 5' end.

The reaction is then boiled for one minute to denature the RNA:DNA duplex. The reaction is then cooled to 420C over one minute. During this cooling step, the target-complementary segment of primer A hybridizes to the
DNA strand synthesized in the previous step. At the same time, primer B hybridizes to its complementary sequence on the RNA transcript synthesized in the previous step.

After cooling, 10 units of AMV reverse transcriptase are added, and further incubation for 10 minutes at 420C is carried out. Reverse transcriptase synthesizes DNA complementary to the HIV target using oligonucleotide A as a primer at the 5' end and DNA made in the previous step as a template. The 3' end of the template strand is used as a primer to produce a final duplex DNA with a double-stranded, T7 promoter site at its 5' end. A second DNA strand is synthesized using oligonucleotide B as a primer and the RNA transcript made in the previous step as a template.

One hundred units of T7 polymerase are added to the reaction. The reaction is incubated for 30 minutes at370 C. The T7 RNA polymerase synthesizes an RNA transcript using the duplex DNA containing the polymerase binding site (T7 promoter) at its 5' end as a template. The RNA transcript is complementary to the target second subsegment, see Figure 1, containing the additional sequence 5'-GGGA-3' at its 5' end. Further cycles may be performed to increase the amplification. The resulting products can be detected by a nucleic acid hybridization protocol.

EXAMPLE X
Protocol for Last-Round Labeling of a TAS Amplified
Product
A. Column to remove unincorporated cold nucleotides
1. The TAS reaction (100pl) is taken after the cDNA synthesis in the final cycle of TAS. Add 400jil of Reagent A (0.1 M Tris-HCl, pH 7.7, 10 mM triethylamine, 1 mM disodium EDTA) to the reaction.

2. Equilibrate a NENSORB20TM (Dupont) prepac column by wetting the column material in1008 methanol (HPLC grade), then equilibrating with 2 ml Reagent A.

3. Load sample on column.

4. Wash the column 3 times with 1 ml Reagent A.

5. Wash the column 3 times with 1 ml water.

6. Elute the nucleic acids with 50% methanol, collecting 250-300jil fractions up to 1 ml total volume.

(The first two fractions will contain the majority of the nucleic acid.)
7. Dry the fractions in the speed-vac or lyophilizer.

B. Protocol to Label the Amplified Product
1. Resuspend the fractions from the NENSORB20TM column (the first two fractions may be combined) in 40 mM
Tris-HCl, pH 8.1, 8 mMMgC12, 25 mM NaCl, 2 mM spermidine (HCl)3, 5 mM dithiothreitol, 400pM each rATP, rCTP, and rGTP, 12HM rUTP, and 25pCi a-32p-rUTP (800 Ci/mmol).

2. Add 50 units T7 RNA polymerase (New England
Biolabs) for each 50jil of sample. Incubate at 370C for 30 minutes.

3. The unincorporated label may be removed by a
G-50 spin column (Maniatis supra).

4. The labeled sample may be run on a sequencing polyacrylamide gel to determine the size of the amplified product. The labeled product may also be detected using theOligo-BeadsTM.

EXAMPLE XI
Use of an Internal Standard During TASAmtlification
Samples were prepared having: 1) 0.1 fm HIV RNA and 0.1 fmB-globin DNA sequences(HB19A cleaved with
PstI); 2) 0.01 fm HIV RNA and 0.1 fm p-globin DNA (HP19A cleaved with PstI); 3) 0.1 fm HIV RNA; or 4) 0.1 fmp- globin DNA(HB19A cleaved with PstI in 100jil containing 40 mM Tris-HCl, pH 8.1, 8 mMMgC12, 25 mM NaCl, 2 mM spermidine-(HCl)3, 5 mM dithiothreitol, 1001 each dATP, dTTP, dCTP and dGTP, 1 mM each rATP, rUTP, rCTP, and rGTP, 100pg/ml BSA (nuclease-free) and 250 nM primer A (5' AATTTAATACGACTCACTATAGGGACACCTAGGGCTAACTATGTGTCCTAATAAGG- 3'), 250 nM primer B (5'-ACACCATATGTATGTTTCAGGGAAAGCTA-3'), 250 nM primer C (5 '-TAATACGACTCACTATAGGGAACTAAAGGCACCGAGCACTTTCTTGCC-3'), and 250 nM primer D (5'-ACATTGCTTCTGACACAACTGTGTTCA-3').

A sample having 1/20th the starting nucleic acids was placed into denaturing solution7.4t formaldehyde, lOx SSC (1.5 M NaCl,0.15 M Na citrate, pH 7.4) for the zero time-point samples.

The samples were boiled for 1 minute and cooled at 420C for 1 minute, followed by the addition of 10 units of
AMV reverse transcriptase. The reactions are incubated at 420C for 15 minutes, boiled for 1 minute, and cooled at 420C for 1 minute. An additional 10 units of AMV reverse transcriptase are added, followed by incubation at 420C for 15 minutes. One hundred units of T7 RNA polymerase are added, followed by incubation at 370C for 30 minutes.

This cycle is repeated a second time. (More cycles can be done, depending on the amplification needed.) Two samples containing 1/20th the starting target nucleic acids were placed into denaturing solution for the second transcription time-point samples.

All samples were heated to 550C for 30 minutes prior to filtering onto two nitrocellulose membranes. The nucleic acids were fixed to the membrane by irradiation with 254 nm UV light for 4 minutes. As controls, 1, 0.1, and 0.01 fm of plasmid pARV7/2 [Luciw et al., Nature 312, 760 (1984)], which contains the entire HIV genome cDNA as well as plasmidHpl9A [Wallace et al., Nucl. Acids Res. 9, 3647 (1981)], were denatured in 0.2 N NaOH, neutralized with an equal volume of 2 M ammonium acetate, and then filtered onto the nitrocellulose membrane. One membrane was hybridized with 32p-labeled oligonucleotide86-311, which is specific for the amplified product of HIV.The other membrane was hybridized to 32p-labeled oligonucleotide 87-4592, which will hybridize to the amplified p-globin product, as well as to the targetss

globin plasmid.

The hybridizations were in 1% SDS, 0.9 M NaCl, 50 nM NaH2PO4 (pH 7.4), 5 mM EDTA (pH 7.4), and 106 cpm/m.

32p-labeled oligonucleotide for 1 hour at 550C. The membranes were washed 3 times in 1% SDS, 0.18 M NaCl, 10 mM NaH2P04 (pH 7.4), and 1 mM EDTA at room temperature, followed by 1 wash in the same buffer at 550C.

The membranes were then autoradiographed for 16 hours on Kodak XAR film at -700C with one intensifying screen.

Notes 1. 86-31: 5'-GCACACAAGTAGACCCTGAACTAGCAGACCA-3' 2. 87-459:5' - AGGTTTAAGGAGACCAATAGAAACT-3'
The autoradiograph in Figure 3 shows the amount of
HIV and p-globin sequences detected after two cycles of
TAS carried out simultaneously on HIV andB-globin nucleic acid. The starting amount of p-globin was kept constant while the amount of HIV target was varied.

While the invention has been described with some specificity in the present specification, persons of ordinary skill in the pertinent arts will recognize variations and modifications of the invention as described that are within the spirit of the invention. Such variations and modifications are also within the scope of the invention as described and claimed herein.

TRANSCRIPTION-BASED NUCLEIC ACID AMPLIFICATION/DETECTION SYSTEMS

Claims of **WO8810315**

WHAT IS CLAIMED IS:

1. A method of preparing a double-stranded nucleic acid containing a sequence corresponding to a target sequence operably linked to a promoter therefor, comprising:
providing a first nucleic acid primer containing a promoter sequence operably linked to a sequence corresponding to a segment of a target sequence,
hybridizing under suitable conditions said first nucleic acid primer with target sequence in a sample containing nucleic acid,
extending the hybridized said first nucleic acid primer in a polymerase extension reaction complementarily to the target sequence to form a corresponding duplex nucleic acid,
separating the strands of said duplex,
hybridizing to the separated promoter containing sequence strand under suitable conditions a second nucleic acid primer at the end opposite from said promoter sequence containing strand, and
extending the hybridized said second nucleic acid primer in a polymerase extension reaction complementarily to said promoter sequence containing strand.

2. The method according to Claim 1 repeated at least once.

3. A method useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising employing the doublestranded nucleic acid according to Claim 1 as a doublestranded nucleic acid template for the preparation of a plurality of RNA transcripts therefrom, each bearing an
RNA sequence corresponding to said target sequence, and detecting the presence of said RNA sequence.

4. A method of preparing a double-stranded nucleic acid containing a sequence corresponding to a target sequence operably linked to a promoter therefor, comprising providing a first nucleic acid primer containing a promoter sequence operably linked to a sequence complementary to a segment of a target sequence and a second nucleic acid primer having a sequence identical to a segment of a target sequence, said primers corresponding to different regions of said target sequence but not, or not substantially, overlapping in their correspondence to the target and being selected such that an extension product of one when separated from its complement can serve as a template for an extension product of the other, contacting a sample containing nucleic acid including target sequence with said primers under sequential hybridizing and strand separation conditions so as to produce, in turn, extension products of said primers.

5. A method comprising employing the doublestranded nucleic acid according to Claim 1 or 4 as a template for the preparation of a plurality of RNA transcripts therefrom in a reaction catalyzed by a polymerase that recognizes the promoter thereof, and detecting the presence of said RNA transcripts.

6. The method according to Claim 3 or 5 wherein said transcripts contain replicase recognition site for replication of said transcripts by replicase induction.

7. The method according to Claim 3, 5 or 6 wherein the detected RNA sequence of said RNA transcripts are measured in a standardized manner so as to measure the amount of target sequence contained in a sample of nucleic acid used in preparing the double-stranded nucleic acid template.

8. The method according to Claim 7 wherein the detected RNA sequence of said RNA transcripts is measured in a manner internally standardized with the presence of a known copy number of nucleic acid also contained in said sample.

9. The method according to Claim 3 wherein said target sequence is disposed within a nucleic acid sequence associated with the characteristics of a genetic or pathogenic disease or condition.

10. The method according to Claim 9 wherein said nucleic acid sequence is a segment of a human immunodeficiency virus.

11. A method according to Claim 10 wherein said nucleic acid sequence is a segment of a defective gene.

12. A method according to Claim 5 wherein the promoter is a bacteriophage T7 promoter and the RNA transcripts are produced using T7 RNA-polymerase.

13. A method according to Claim 5 wherein the promoter is a SP6 promoter and the RNA transcripts are produced using SP6 RNA polymerase.

14. A method according to Claim 1 or 4 wherein the extension reaction is catalyzed by E. coli DNA polymerase I.

a 15. A method according to Claim 1 or 4 wherein the extension reaction is catalyzed by Klenow fragmentof -E.

coli DNA polymerase I.

16. A method according to Claim 1 or 4 wherein the extension reaction is catalyzed by T4 DNA polymerase.

17. A method according to Claim 1 or 4 wherein the extension reaction is catalyzed by reverse transcriptase.

18. A method according to Claim 3 or 5 wherein said RNA transcripts are labelled prior to detection.

19. A method according to Claim 18 wherein said
RNA transcripts are radio-labelled.

20. A method according to Claim 18 wherein said
RNA transcripts are chromophore labelled.

21. A kit useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising a first nucleic acid primer containing a promoter sequence operably linked to a sequence complementary to a segment of a target sequence and a second nucleic acid primer having a sequence identical to a segment of a target sequence, said primers corresponding to different regions of said target sequence but not, or not substantially, overlapping in their correspondence to the target and being selected such that an extension product of one when separated from its complement can serve as a template for an extension product of the other, and means for hybridizing said first primer to said target sequence, chain extending the hybridized primer, strand-separating the resultant duplex, hybridizing to the promoter containing separated strand said second nucleic acid primer, chain extending the hybridized primer, causing the thus prepared doublestranded nucleic acid containing a promoter sequence to produce transcripts, and detecting said transcripts.

22. A method for amplifying a target nucleic acid segment of Formula I 3'-(first subsegment)t-(second subsegment)t -(third subsegment)t-5'
I wherein (first subsegment)t is a nucleic acid segment of known sequence of at least 10 nucleotides adjoining the 3'-terminus of (secondsubsegment)tt (second subsegment)t is a nucleic acid segment ofo or more nucleotides, and (third subsegment)t is a nucleic acid segment of known sequence of at least 10 nucleotides adjoining the 5'-terminus of (secondsubsegment)t, which method comprises::
(1) hybridizing to (first subsegment)t of said target segment a first primer, which is a single-stranded DNA which comprises a 3'-terminal subsegment of Formula II 5'-(promoter)I-(variablesubsegment) 1 (3'-primersubsegment)1-3' wherein (promoter)I is a single-stranded DNA segment with the sequence of the plus-strand of a bacteriophage
DNA-dependent RNA polymerase-specific promoter, (variable subsegment)I is a single-stranded DNA segment of0 to 100 nucleotides adjoining the 3'-terminal nucleotide of (promoter)I and the 5'-terminal nucleotide of (3'-primer subsegment)I, and (3'-primer subsegment)I is a single-stranded DNA segment of at least 10 nucleotides with a sequence which is complementary to the sequence of a subsegment of (first subsegment)t which terminates with the 3'-terminal nucleotide of (firstsubsegment)t, said (promoter)I

adjoining the 5'-terminus of said (3'-primer subsegment)l, if said (variable subsegment)l has 0 nucleotides;;
(2) extending said first primer, hybridized in accordance with step (1), in a reaction catalyzed by a first DNA polymerase to make a first complementary DNA segment, which comprises a subsegment of Formula III 5'-(promoter)l-(variable subsegment)l (firstsubsegment) tc (second subsegment) tc (thirdsubsegment)tc-3 ,
III wherein (firstsubsegment)te is the DNA segment with the sequence complementary to that of (firstsubsegment)t, (secondsubsegment)tc is the DNA segment with the sequence complementary to that of (secondsubsegment)t, and (thirdsubsegment)tc is the DNA segment with the sequence complementary to that of (thirdsubsegment)t, provided that, if the target segment is an
RNA segment, said first DNA polymerase is a reverse transcriptase;
(3) rendering single-stranded the duplex formed in the reaction of step (2);
(4) hybridizing to (thirdsubsegment)tc of said first complementary DNA of Formula III a second primer, which is a single-stranded DNA of at least 10 nucleotides of Formula IV3 '-(5'-primer subsegment)2-(variablesubsegment)2-5',
IV wherein (5'-primer subsegment)2 has the sequence of a subsegment of (third subsegment)t which terminates with the 5'-terminal nucleotide of (thirdsubsegment) and wherein (variable subsegment)2 is a segment of0 to 100 nucleotides which adjoins the 5'-terminus of (5'-primer subsegment)2;;
(5) extending said second primer segment, hybridized in accordance with step (4), in a reaction catalyzed by a second DNA polymerase to form a second complementary DNA segment which comprises a subsegment of
Formula V5'- (variable subsegment) 2-(third subsegment)t (second subsegment)t-(first subsegment)t (variablesubsegment)lc-(promoter)lc-3',
V wherein (variablesubsegment)1 is the DNA segment with the sequence complementary to that of (variablesubsegment)l, and(promoter) 1c is the DNA segment with the sequence complementary to that of (promoter)l, provided that said second DNA polymerase is the same-as or different from said first DNA polymerase; and
(6) employing the double-stranded product of step (5) as the template in a reaction catalyzed by a first bacteriophage DNA-dependent RNA polymerase, which recognizes the promoter of which one strand is (promoter)1, to make a first RNA product of Formula VI5'- (variable subsegment)lr-(first subsegment)tcr-(secondsubsegment) tcr (third subsegment)tc,- (variablesubsegment) 2cr3,
VI wherein (variable subsegment)lr is the RNA segment with the sequence of (variable subsegment)l, (first subsegment)tcr is the RNA segment with the sequence complementary to that of (firstsubsegment)t, (second subsegment)tcr is the RNA segment with the sequence complementary to that of (secondsubsegment)t, (third subsegment)tcr is the RNA segment with the sequence complementary to that of (thirdsubsegment)t, and (variable subsegment)2cr is the RNA segment with the sequence complementary to that of (variable subsegment)2.

23. A method according to Claim 22 wherein (first subsegment)t is at least 10 nucleotides in length and is of Formula XIII 3'-(first subsegment)t2-(firstsubsegment)tl-5',
XIII wherein (first subsegment)t2 has 0 or more nucleotides and, if more than 0, adjoins the 3'-terminus of (firstsubsegment)tl, and (first subsegment)tl is at least 10 nucleotides in length; wherein (third subsegment)t is of
Formula XIV3,- (third subsegment)tl-(third subsegment)t2-5',
XIV wherein (third subsegment)t2 has 0 or more nucleotides and, if more than 0, adjoins the 5'-terminus of (thirdsubsegment)tl, and (third subsegment)tl is at least 10 nucleotides in length; wherein (3'-primer subsegment)l has the sequence complementary to that of a subsegment of target segment which consists of all of
(first-subsegment)t2 and 0 or more nucleotides of (firstsubsegmenttl, wherein
(5'-primer subsegment)2 is a subsegment which consists of all of (third subsegment)t2 and 0 or more nucleotides of
(thirdsubsegment)tl; wherein (variable subsegment)2 has 0 nucleotides; and wherein, after steps (1) to (6) of Claim 1, the RNA subsegment of Formula VII5'-(first subsegment) tlcr (second subsegment)tcr~(thirdsubsegment)tlcr~3
VII wherein (firstsubsegment)tlcr is the RNA segment with the sequence complementary to that of (first subsegment)tl and (thirdsubsegment)tlcr is the RNA segment with the sequence complementary to that of (thirdsubsegment)tl, is further amplified by a method which comprises: :
(7) hybridizing to said first RNA product of
Formula VI a third primer, which is a single-stranded DNA which comprises a 3'-terminal subsegment of Formula VIII5'- (promoter) 3 (variable subsegment)3-
(3 '-primer subsegment)3-3',
VIII wherein (promoter)3 is a single-stranded DNA segment with the sequence of the plus-strand of a

bacteriophage
DNA-dependent RNA polymerase-specific promoter, said sequence of (promoter)3 being the same as or different from that of (promoter)l,
(variable subsegment)3 is a single-stranded DNA segment of0 to 100 nucleotides which adjoins the 3'-terminal nucleotide of (promoter)3 and the 5'-terminal nucleotide of (3'-primer subsegment)3, and (3'-primer subsegment)3 is a single-stranded DNA segment which has the same sequence as (third subsegment)tl and adjoins the 3'-terminal nucleotide of (promoter)3 if (variable subsegment)3 has 0 nucleotides;
(8) extending said third primer hybridized in accordance with step (7) in a reaction catalyzed by a third DNA polymerase, which is a reverse transcriptase and is the same as or different from said first and second DNA polymerase, to make a third complementary DNA segment which comprises a 3'-terminal subsegment of Formula IX 5'-(promoter)3-(variable subsegment)3 (thirdsubsegment)tl-(second subsegment)t (first subsegment)tl-(first subsegment)t2 (variablesubsegment)lc-3',
IX wherein (variable subsegment)lc is the DNA with the sequence complementary to that of (variablesubsegment)l;;
(9) rendering single-stranded the duplex formed in the reaction of step (8);
(10) hybridizing to the third complementary DNA made in the reaction of step (8) a fourth primer of Formula X 5'-(variable subsegment)4-(5'-primer subsegment)4'3
X wherein (variable subsegment)4 is a segment of <RTI 0 to 100 nucleotides, and (5'-primer subsegment) 4 is a subsegment of known sequence which adjoins the 3'-nucleotide of (variable subsegment)4, if (variable subsegment)4 has more than 0 nucleotides, and which comprises at least 10 nucleotides of the segment of Formula XX5'- (variable subsegment)1-(firstsubsegment)t2c- (first subsegment)t1c-3',
XX wherein (first subsegment)t2c is the DNA segment with the sequence complementary to that of (first subsegment)t2 and (firstsubsegment)tlc is the DNA segment with the sequence complementary to that of (first subsegment)tl ' provided that at least one of said at least 10 nucleotides is at or 5' from the 5'-terminal nucleotide of (firstsubsegment)tlc;;
(11) extending said fourth primer hybridized in accordance with step (10) <RTI in a reaction catalyzed by a fourth DNA polymerase, which is the same as or different from said first, second and third DNA polymerase, to form a fourth complementary DNA which comprises a segment of
Formula XI5'- (first subsegment)tlc-(secondsegment)tC- (thirdsubsegment) tlc (variable subsegment)3c (promoter)3c-3' r
XI wherein (secondsubsegment)tc is the DNA segment with the sequence complementary to that of (secondsubsegment)t, (third subsegment)t1c is the DNA segment with the sequence complementary to that of (thirdsubsegment)tlt (variablesubsegment)3 is the DNA segment with the sequence complementary to that of (variable segment)3, and (promoter)3c is the DNA segment with the sequence complementary to that of (promoter)3; and
(12) employing the double-stranded product of step (11) as the template in a reaction catalyzed by a second bacteriophage DNA-dependent RNA polymerase, which is the same as or different from said first bacteriophage
DNA-dependent RNA polymerase and which recognizes the promoter of which one strand is (promoter)3, to make a second RNA product with a 5'-terminal subsegment of
Formula XII5'- (variable subsegment) 3r-(third subsegment)tl,- (secondsubsegment)tr-(first subsegment) tlr X12- (variable subsegment)4cr-3/
XII wherein (variable subsegment)31 is the RNA segment with the sequence of (variable subsegment)3, (third subsegment)tlr is the RNA segment with the sequence of (thirdsubsegment)tl, (secondsubsegment) tr is the RNA segment with the sequence of (secondsubsegment)t, (firstsubsegment)tlr is the RNA segment with the sequence of (firstsubsegment)tl, (variable subsegment)4cr is the RNA segment with the sequence complementary to that of (variable subsegment)4, and X12 is the RNA segment with the sequence complementary to that of the subsegment of (5'-primer subsegment)4 that is 5' from the 5'-terminus of (firstsubsegment)tlc.

24. A method according to Claim 22 wherein each of (variable subsegment)l and (variable subsegment)2 has 0 to 60 nucleotides, each of (3'-primer subsegment)l and (5'-primer subsegment)2 has 15 to 45 nucleotides, (second subsegment)t has at least 30 nucleotides, and target segment has 1000 or fewer nucleotides.

25. A method according to Claim 23 wherein the target segment is a DNA segment, wherein the product of step(2) is rendered single-stranded by thermal denaturation, wherein (variable subsegment)2 has 0 nucleotides, wherein each of the first and second DNA polymerase is selected from the group consisting of Klenow
Fragment of E. coli DNA polymerase I, AMV reverse transcriptase, cloned MMLV reverse transcriptase,

calf thymus DNA polymerase alpha, Thermus aquaticus heat-stable
DNA polymerase, and SequenaseTM brand cloned T7 DNA polymerase, and wherein the bacteriophage DNA-dependent
RNA polymerase is selected from the group consisting of T7
RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase.

26. A method according to Claim 25 wherein each of the first and second DNA polymerase is selected from the group consisting of Klenow Fragment of E. coli DNA polymerase I, AMV reverse transcriptase, and cloned MMLV reverse transcriptase.

27. A method according to Claim 26 wherein (1) the bacteriophage DNA-dependent RNA polymerase is T7 RNA polymerase, (promoter)l is 5'-TAATACGACTCACTATA-3' and (variable subsegment)l has a dinucleotide of sequence 5'-GG-3' at its 5'-terminus; or (2) the bacteriophage
DNA-dependent RNA polymerase is T3 RNA polymerase, (promoter)l is 5'-TATTAACCCTCACTAAA-3' and (variable subsegment)l has a tetranucleotide of sequence 5'-GGGA-3'at its 5'-terminus; or (3) the bacteriophage
DNA-dependent RNA polymerase is SP6 RNA polymerase, (promoter)l is 5'-
GAACGCGGCTACAATTAATACATAAC
CTTATGTATCATACACATACGATTTAGGTGACACTATA-3', and (variable subsegment)l has a hexanucleotide of sequence 5'-GAATAC-3' at its 5'-terminus.

28. A method according to Claim 27 wherein the 5'-terminus of the first primer is the 5'-nucleotide of (promoter)l.

29. A method according to Claim 28 wherein, if the bacteriophage DNA-dependent RNA polymerase is the T7 or T3
RNA polymerase, (variable subsegment)l has the sequence5 '-
GGGATGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3' and, if the bacteriophage DNA-dependent RNA polymerase is the SP6 RNA polymerase, (variable subsegment)l has the sequence5' -
GAATACTGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC- 3,.

30. A method according to Claim 24 wherein the target segment is a RNA segment, wherein the product of step(2) is rendered single-stranded by thermal denaturation, wherein (variable subsegment)2 has 0 nucleotides, wherein the first DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned
MMLV reverse transcriptase, wherein the second DNA polymerase is selected from the group consisting of Klenow
Fragment of E. coli DNA polymerase I, AMV reverse transcriptase, cloned MMLV reverse transcriptase, calf thymus DNA polymerase alpha, Thermus aquaticus heat-stable DNA polymerase, and SequenaseTM brand cloned
T7 DNA polymerase, and wherein the bacteriophage
DNA-dependent RNA polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase and SP6
RNA polymerase.

31. A method according to Claim 30 wherein the second DNA polymerase is selected from the group consisting of Klenow Fragment of E. coli DNA polymerase I,
AMV reverse transcriptase, and cloned MMLV reverse transcriptase.

32. A method according to Claim 31 wherein (1) the bacteriophage DNA-dependent RNA polymerase is T7 RNA polymerase, (promoter)l is 5'-TAATACGACTCACTATA-3' and (variable subsegment)l has a dinucleotide of sequence 5'-GG-3' at its 5'-terminus; or (2) the bacteriophage
DNA-dependent RNA polymerase is T3 RNA polymerase, (promoter)l is 5' -TATTAACCCTCACTAAA-3' and (variable subsegment)l has a tetranucleotide of sequence 5'-GGGA-3'at its 5'-terminus; or (3) the bacteriophage
DNA-dependent RNA polymerase isSP6 RNA polymerase, (promoter)l is 5'-
GAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACGATTTAGGTGACACTATA-3', and (variable subsegment)l has a hexanucleotide of sequence5'-GAATAC-31 at its 5'-terminus.

33. A method according to Claim 32 wherein the 5'-terminus of the first primer is the 5'-nucleotide of (promoter) 1

34. A method according to Claim 33 wherein each of the first and second DNA polymerase is selected from AMV reverse transcriptase and cloned MMLV reverse transcriptase.

35. A method according to Claim 34 wherein, if the bacteriophage DNA-dependent RNA polymerase is the T7 or T3
RNA polymerase, (variable subsegment)l has the sequence5' - GGGATGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3' and, if the bacteriophage DNA-dependent RNA polymerase is the SP6 RNA polymerase, (variable subsegment)l has the sequence5' - GAATACTGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3'
36.A method according to Claim 30, 31, 32, 33, 34 or 35 wherein the target segment is a segment of the genome of a human immunodeficiency virus that is an HIV-1 virus and wherein (1) (3'-primer subsegment) l has the sequence5'-TCTAATTACTACCTCTTCTTCTGCTAGACT-3' and (5'primer CCTCTTCTTCTGCTAGACT-3' and (5'-primer subsegment)2 has the sequence5'-ACAAGTTGTAACACCTCAGTCATTACACAG-3' or (2) (3'-primer subsegment)l has the sequence5' - TTTCGTAACACTAGGCAAAGGTGGCTTTATC-3' and (5'-primer subsegment)2 has a sequence selected from the group consisting of5'-GCACACAAGTAGACCCTGAACTAGCAGACCA-3' and5' - ACACCATATGTATGTTTCAGGGAAAGCTA-3'.

37. A method according to Claim 23 wherein each of (variable subsegment)l and (variable subsegment)3 has 0 to 60 nucleotides, wherein each of (3'-primersubsegment)1 (5 '-primer subsegment)2,(3 '-primer subsegment)3, and (5'-primer subsegment)4 has 15 to 45 nucleotides, (second subsegment)t has at least 30 nucleotides, and target segment has 1000 or fewer nucleotides.

38. A method according to Claim 37 wherein (A) if the target segment is a DNA segment, the product of step (2) is rendered single-stranded by thermal denaturation and the product of step (8) is rendered single-stranded by thermal denaturation; each of the first, second and fourth
DNA polymerase is selected from the group consisting of
Klenow Fragment of E. coli DNA polymerase I, AMV reverse transcriptase, cloned MMLV reverse transcriptase, calf thymus DNA polymerase alpha, Thermus aquaticus heat-stable
DNA polymerase, and SequenaseTM brand cloned T7 DNA polymerase; the third DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned
MMLV reverse transcriptase; and the each of the first and second bacteriophage DNA-dependent RNA polymerase is selected from the group consisting of T7 RNA polymerase,
T3 RNA polymerase and SP6 RNA polymerase; and (B) if the target segment is a RNA segment, each of the product of step (2) and the product of step (8) is rendered single-stranded by thermal denaturation; each of the second and fourth DNA polymerase is selected from the group consisting of Klenow Fragment of E. coli DNA polymerase I, AMV reverse transcriptase, cloned MMLV reverse transcriptase, calf thymus DNA polymerase alpha,
Thermus aquaticus heat-stable DNA polymerase, and
SequenaseTM brand cloned T7 DNA polymerase; each of the first and third DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned MMLV reverse transcriptase; and the each of the first and second bacteriophage DNA-dependent RNA polymerase is selected from the group consisting of T7 RNA polymerase,
T3 RNApoymerase and SP6 RNA polymerase.

39. A method according to Claim 38 wherein the target segment is a RNA segment and wherein each of the second and fourth DNA polymerase is selected from the group consisting of Klenow Fragment of E. coli DNA polymerase I, AMV reverse transcriptase , and cloned MMLV reverse transcriptase and wherein each of the first and third DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned MMLV reverse transcriptase.

40. A method according to Claim 38 wherein the subsegment of target segment which has the sequence complementary to that of (3'-primer subsegment)l is in the 3'-direction from and does not overlap the subsegment of target segment which has the sequence complementary to that of (5'-primer subsegment) 4, and wherein the subsegment of target segment which has the sequence of (5'-primer subsegment)2 does not overlap the subsegment of target segment which has the sequence of (3'-primer subsegment)3.

41. A method according to Claim 40 wherein the subsegment of third complementary DNA with the sequence complementary to that of (3'-primer subsegment)l is in the 5'-direction from and does not overlap the subsegment of third complementary DNA which has the sequence complementary to that of (5'-primer subsegment)4, and wherein the subsegment of target segment which has the sequence of (5'-

primer subsegment)2 does not overlap the subsegment of target segment which has the sequence of (3'-primer subsegment)3.

42. A method according to Claim 41 wherein, in the third complementary DNA, (variablesubsegment)1 has more than 0 nucleotides and wherein, in the duplex between(57-primer subsegment)4 and third complementary DNA, at least a subsegment of (5'-primer subsegment)4 is hybridized to (variablesubsegment)lc.

43. A method according to Claim 41 wherein the subsegment of target segment which has the sequence complementary to that of (3'-primer subsegment)l is in the 3'-direction from and does not overlap the subsegment of target segment which has the sequence complementary to that of (5'-primer subsegment) 4, and wherein the subsegment of target segment which has the sequence of (5'-primer subsegment)2 does not overlap the subsegment of target segment which has the sequence of (3'-primer subsegment)3.

44. A method according to Claim 41 wherein the subsegment of third complementary DNA with the sequence complementary to that of (3'-primer subsegment)l is in the 5'-direction from and does not overlap the subsegment of third complementary DNA which has the sequence complementary to that of (5'-primer subsegment)4, and wherein the subsegment of target segment which has the sequence of (5'-primer subsegment)2 does not overlap the subsegment of target segment which has the sequence of(3 '-primer subsegment)3.

45. A method according to Claim 44 wherein, in the third complementary DNA, (variablesubsegment)le has more than 0 nucleotides and wherein, in the duplex between (5'-primer subsegment)4 and third complementary DNA, at least a subsegment of (5'-primer subsegment)4 is hybridized to (variablesubsegment)lc.

46. A method according to Claim 40, 41, 42, 43, 44 or 45 wherein (1) if T7 RNA polymerase is employed in step (6) or step (12), (promoter)l, if the step is step (6), or (promoter)3, if the step is step (12), has the sequence 5'-TAATACGACTCACTATA-3' and (variable subsegment)l, if the step is step (6), or (variable subsegment)3, if the step is step (12), has a dinucleotide of sequence 5'-GG-3' at its 5'-terminus; or (2) if T3 RNA polymerase is employed in step (6) or step (12), (promoter)l, if the step is step (6), or (promoter)3, if the step is step (12), has the sequence 5'-TATTAACCCTCACTAAA-3' and (variable subsegment)l, if the step is step (6), or (variable subsegment)3, if the step is step (12), has a tetranucleotide of sequence 5'-GGGA-3'at its 5'-terminus; or (3) if the SP6 RNA polymerase is employed in step (6) or step (12), (promoter)l, if the step is step (6), or (promoter)3, if the step is step (12), has the sequence5' -GAACGCGGCTACAATTAATACATAAC CTTATGTATCATACACATACGAT-TTAGGTGACACTATA-3', and (variable subsegment)l, if the step is step (6), or (variable subsegment)3, if the step is step (12) has a hexanucleotide of sequence 5'-GAATAC-3' at its 5'-terminus; and wherein the 5'-terminus of the first primer is the 5'-nucleotide of (promoter)l and the 5'-terminus of the third primer is the 5'-nucleotide of(promoter) 3.

47. A method according to Claim 40, 41, 42, 43, 44 or 45 wherein (A) (i) if the first bacteriophage DNA-dependent RNA polymerase is the T7 or T3 RNA polymerase, (variable subsegment)l has the sequence5' -GGGATGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3' and second primer (variable subsegment)2 has the sequence5' -CGCGCTCTCCCAGGTGACGCCTCGAGAAGAGGCGCGACCTTCGTGC-3';; or (ii) if the first bacteriophage DNA-dependent RNA polymerase is the SP6 RNA polymerase, (variable subseg <RTI mental has the sequence 5'-GAATACTGGGGAACCCCCCTTCGG GGGTCACCTCGCGCAGC-3'and second primer (variable subsegment)2 has the sequence5' -CGCGCTCTCCCAGGTGACGCCTCGAGAAGAGGCGCGACCTTCGTGC-3' (B) (i) if the second bacteriophage DNA-dependent RNA polymerase is the T7 or T3 RNA polymerase, (variable subsegment) 3 has the sequence 5'-GGGATGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3'; (ii) if the second bacteriophage DNA-dependent RNA polymerase is the SP6 RNA polymerase, (variable subsegment)3 has the sequence 5-GAATACGGGATGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC3' and the fourth primer has as the 5'terminal 40 nucleotides5'-CGCGCTCTCCCAGGTGACGCCTCGAGAAGAGGCGCGACCTTCGTGC-3'; and (C) after step (12), the second RNA is autocatalytically replicated withQss replicase.

48. A method according to Claim 37, 38, 39, 40, 41, 42, 43, 44 or 45 wherein the target segment is a segment of the genome of a human immunodeficiency virus that is an HIV-1 virus and wherein (1) (3'-primer subsegment)1 has the sequence5' -

TCTAATTACTACCTCTTCTTCTGCTAGACT-3', (5'-primer subsegment)2 has the sequence5' - ACAAGTTGTAACACCTCAGTCATTACACAG-3', (3'-primer subsegment)3 has the sequence 5'- AAAGGTATCCTTTGAGCCAATTCCCATA-3', and fourth primer has the sequence 5'- AGTTGATACTACTGGCCTAATT-3'; or (2) (3'-primer subsegment)l has the sequence5' - TTTCGTAACACTAGGCAAAGGTGGCTTTATC-3', (5 '-primer subsegment)2 has a sequence selected from the group consisting of5' -GCACACAAGTAGACCCTGAACTAGCAGACCA-3' and5' - ACACCATATGTATGTTTCAGGGAAAGCTA-3', (3'-primer subsegment)3 has the sequence 5'- ACTAATTCATCTGTATTACTTTGACTGTTTTTC-3', and fourth primer has the sequence 5'- TTTTTTGGTGTTATTAATGCTGCTAGTGCC-3'.

49. A method according to Claim 24 wherein (A) if the target segment is a DNA segment, the product of step (2) is rendered single-stranded by thermal denaturation; each of the first and second DNA polymerase is selected from the group consisting of Klenow Fragment of E. coli
DNA polymerase I, AMV reverse transcriptase, cloned MMLV reverse transcriptase, calf thymus DNA polymerase alpha,
Thermus aquaticus heat-stable DNA polymerase, and
SequenaseTM brand cloned T7 DNA polymerase; and the first bacteriophage DNA-dependent RNA polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase; and (B) if the target segment is a RNA segment, the product of step (2) is rendered single-stranded by thermal denaturation; the second DNA polymerase is selected from the group consisting of Klenow Fragment of E. coli DNA polymerase I,
AMV reverse transcriptase, cloned MMLV reverse transcriptase, calf thymus DNA polymerase alpha, Thermus aquaticus heat-stable DNA polymerase, and SequenaseTM brand cloned T7 DNA polymerase; the first DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned MMLV reverse transcriptase; and the first bacteriophage DNA-dependent RNA polymerase is selected from the T7 RNA polymerase, the T3 RNA polymerase and the SP6 RNA polymerase.

50. A method according to Claim 49 wherein each of the first and second DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned
MMLV reverse transcriptase.

51. A method according to Claim 50 wherein the 5'-terminus of the first primer is the 5'-nucleotide of (promoter)l; wherein, if the bacteriophage DNA-dependent
RNA polymerase is the T7 RNA polymerase is the T7 RNA polymerase, the subsegment (promoter)l (variable subsegment)l of the first primer has the sequence5' - TAATACGACTCACTATAGGGACGCGCTCTCCCAGGTGACGCCTC-GAGAAGAGGCGCGACCTTCGTGC-3'; wherein, if the bacteriophage
DNA-dependent RNA polymerase is the T3 RNA polymerase, the subsegment(promoter) 1 (variable subsegment)l of the first primer has the sequence5' - TATTAACCCTCACTAAAGGGACGCGCTCTCCCAGGTGACGCCTC-GAGAAGAGGCGCGACCTTCGTGC-3'; wherein, if the bacteriophage
DNA-dependent RNA polymerase is the SP6 RNA polymerase, the subsegment (promoter)l-(variable subsegment)l of the first primer has the sequence 5'- GAACGCGGCTACARTTAATACATAACCTTATGTATCATACACATACGAT-TTAGGTGACACTATAGAATACCGCGCTCTCCCAGGTGACGCCTCGAGAAGAG-GCGCGACCTTCGTGC-3'; wherein (variable subsegment)2 has the sequence 5'- TGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3'; and wherein, after step (6), the first RNA is autocatalytically replicated withQss replicase.

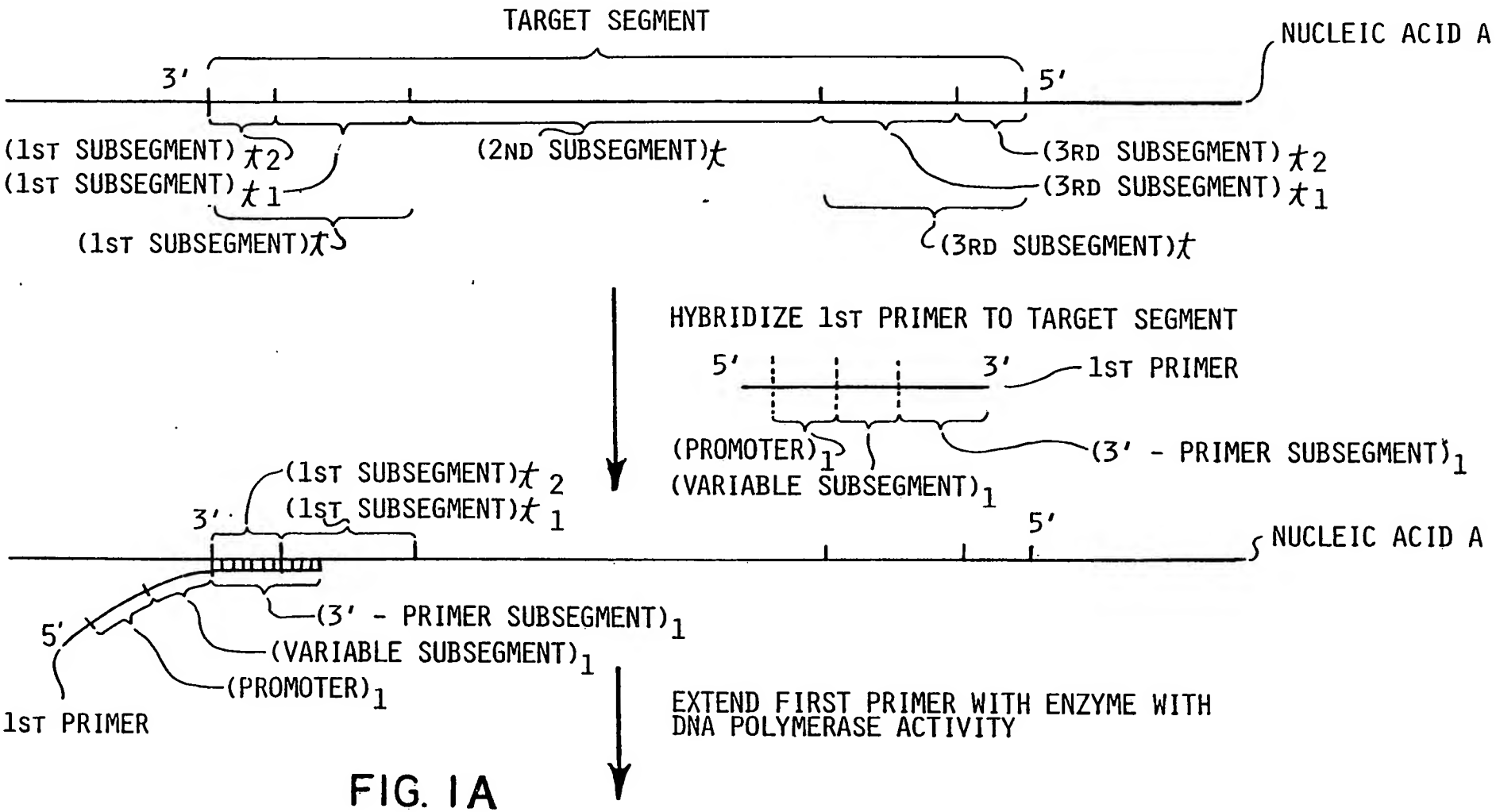


FIG. 1A

PCT/US88/02108

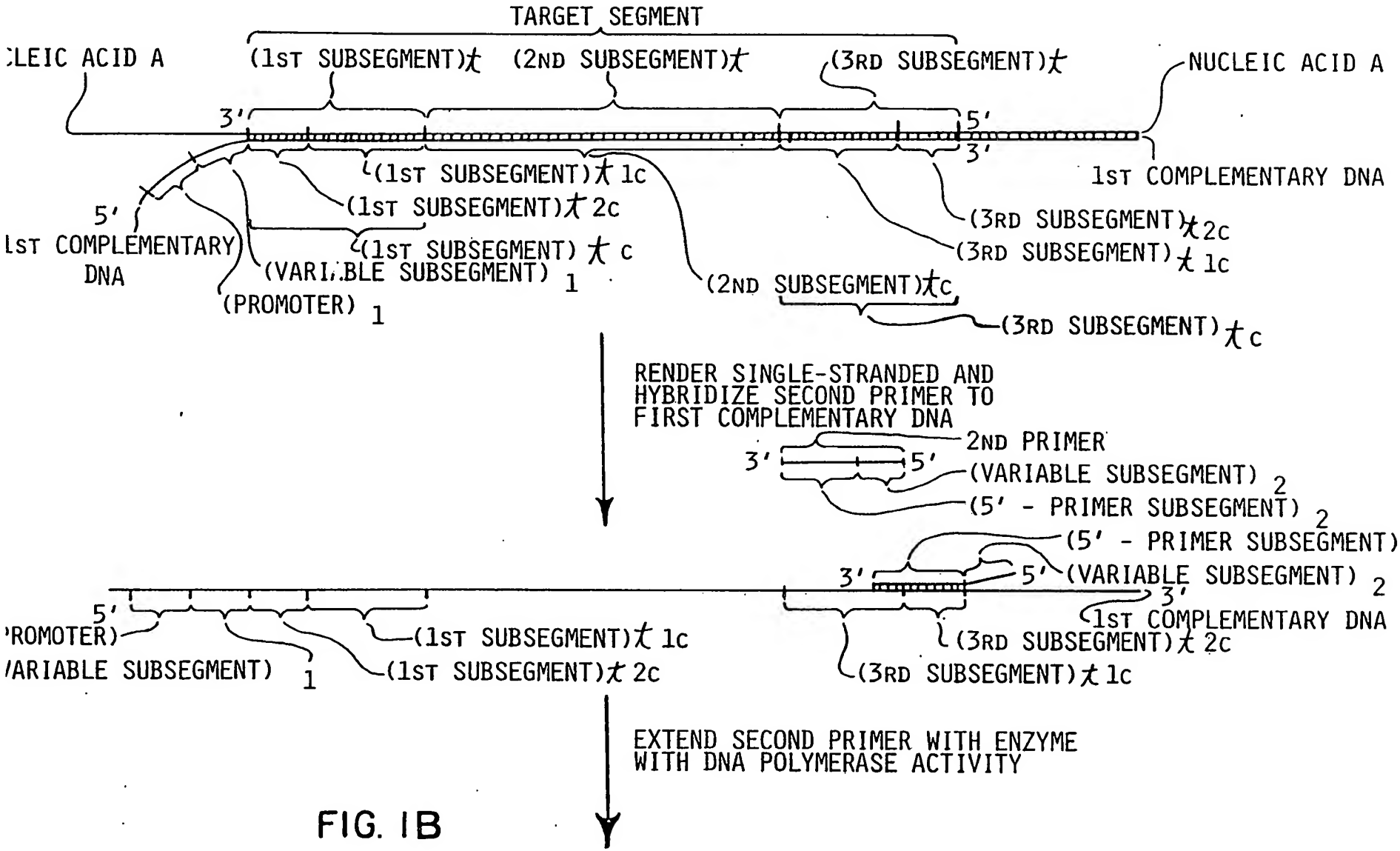

TARGET SEGMENT
LEIC ACID A
(1ST SUBSEGMENT)
(2ND SUBSEGMENT)
(3RD SUBSEGMENT)
NUCLEIC ACID A
3'
5'
3'
(1ST SUBSEGMENT) 1c
(1ST SUBSEGMENT) 2c
(1ST SUBSEGMENT) c
(VARIABLE SUBSEGMENT) 1
(PROMOTER) 1
1ST COMPLEMENTARY DNA
(3RD SUBSEGMENT) 2c
(3RD SUBSEGMENT) 1c
(2ND SUBSEGMENT) c
(3RD SUBSEGMENT) c
5'
1ST COMPLEMENTARY DNA
RENDER SINGLE-STRANDED AND HYBRIDIZE SECOND PRIMER TO FIRST COMPLEMENTARY DNA
2ND PRIMER
3'
5'
(VARIABLE SUBSEGMENT) 2
(5' - PRIMER SUBSEGMENT) 2
(5' - PRIMER SUBSEGMENT) 2
3'
5'
(VARIABLE SUBSEGMENT) 2
3'
1ST COMPLEMENTARY DNA
5'
ROMOTER)
ARIABLE SUBSEGMENT) 1
(1ST SUBSEGMENT) 1c
(1ST SUBSEGMENT) 2c
(3RD SUBSEGMENT) 2c
(3RD SUBSEGMENT) 1c
EXTEND SECOND PRIMER WITH ENZYME WITH DNA POLYMERASE ACTIVITY

FIG. 1B

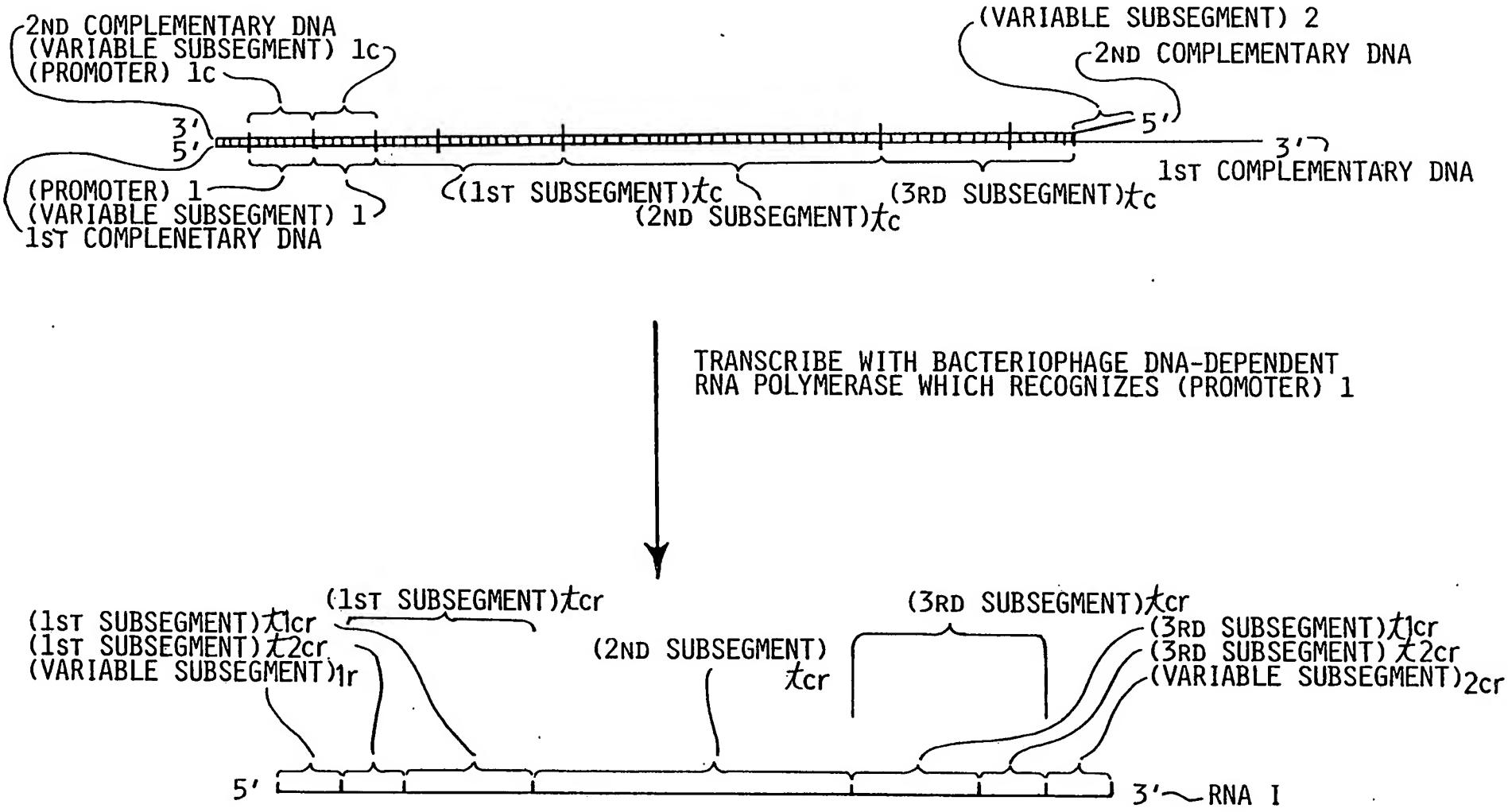


FIG. 1C

PCT/US88/02108

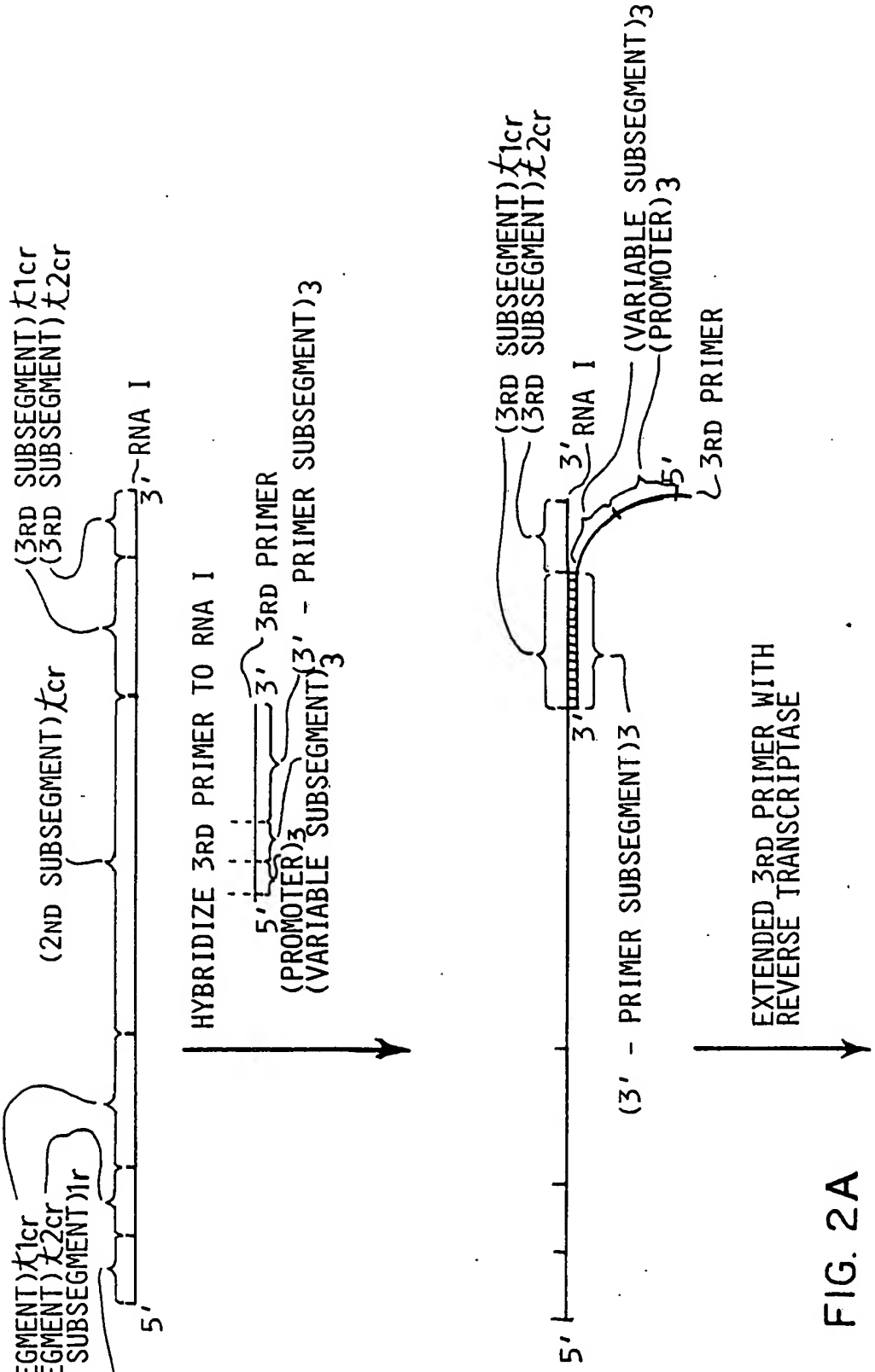

(1ST SUBSEGMENT)ℒ1cr
(1ST SUBSEGMENT)ℒ2cr
(VARIABLE SUBSEGMENT)1r
(2ND SUBSEGMENT)ℒcr
(3RD SUBSEGMENT)ℒ1cr
(3RD SUBSEGMENT)ℒ2cr
3'~RNA I
5'
HYBRIDIZE 3RD PRIMER TO RNA I
5'
(PROMOTER)3
(VARIABLE SUBSEGMENT)3
3' 3RD PRIMER
3' - PRIMER SUBSEGMENT)3
(3RD SUBSEGMENT)ℒ1cr
(3RD SUBSEGMENT)ℒ2cr
3' RNA I
(VARIABLE SUBSEGMENT)3
(PROMOTER)3
5'
3RD PRIMER
3'
3' - PRIMER SUBSEGMENT)3
5'
EXTENDED 3RD PRIMER WITH
REVERSE TRANSCRIPTASE

FIG. 2A

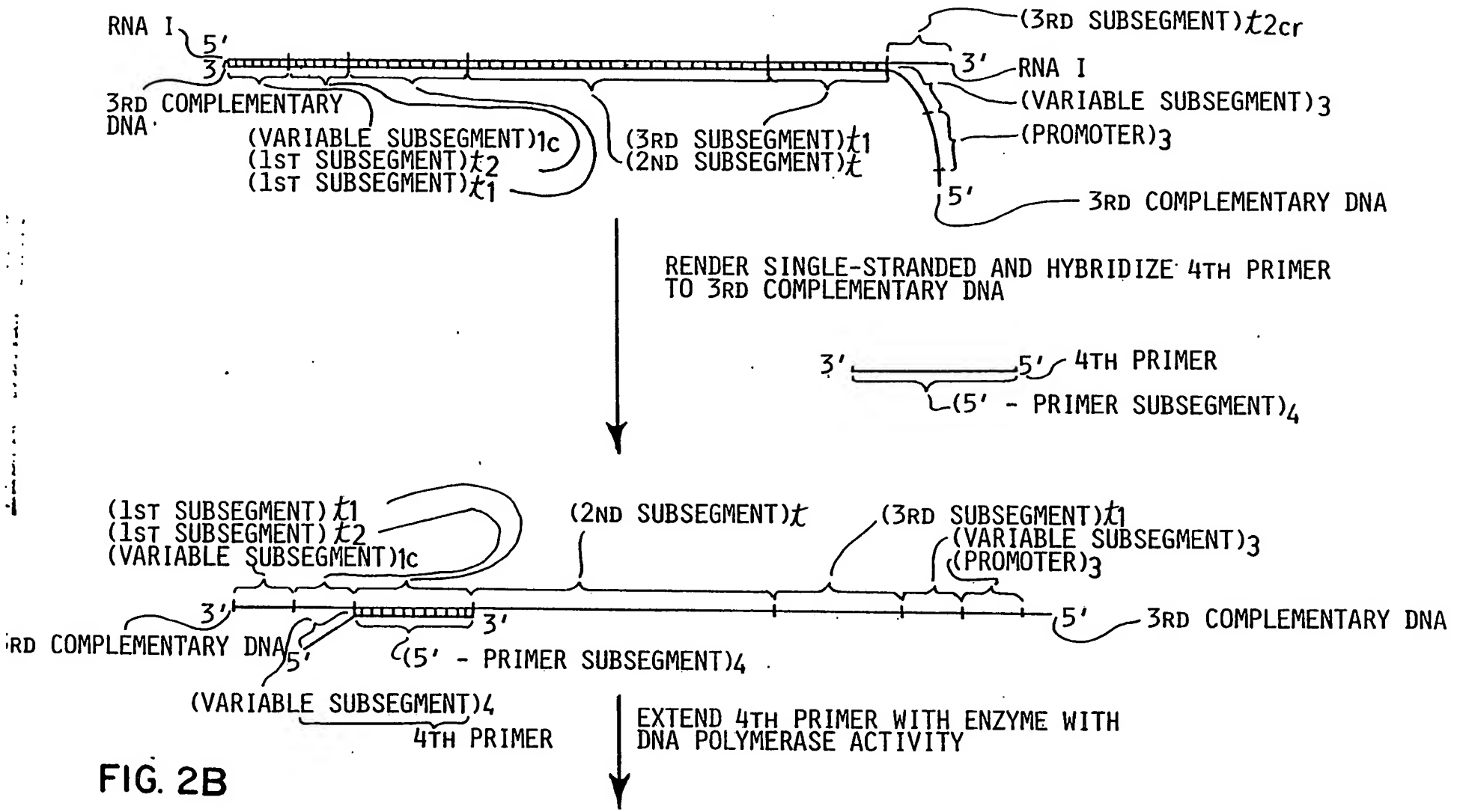


FIG. 2B

PCT/US88/02108

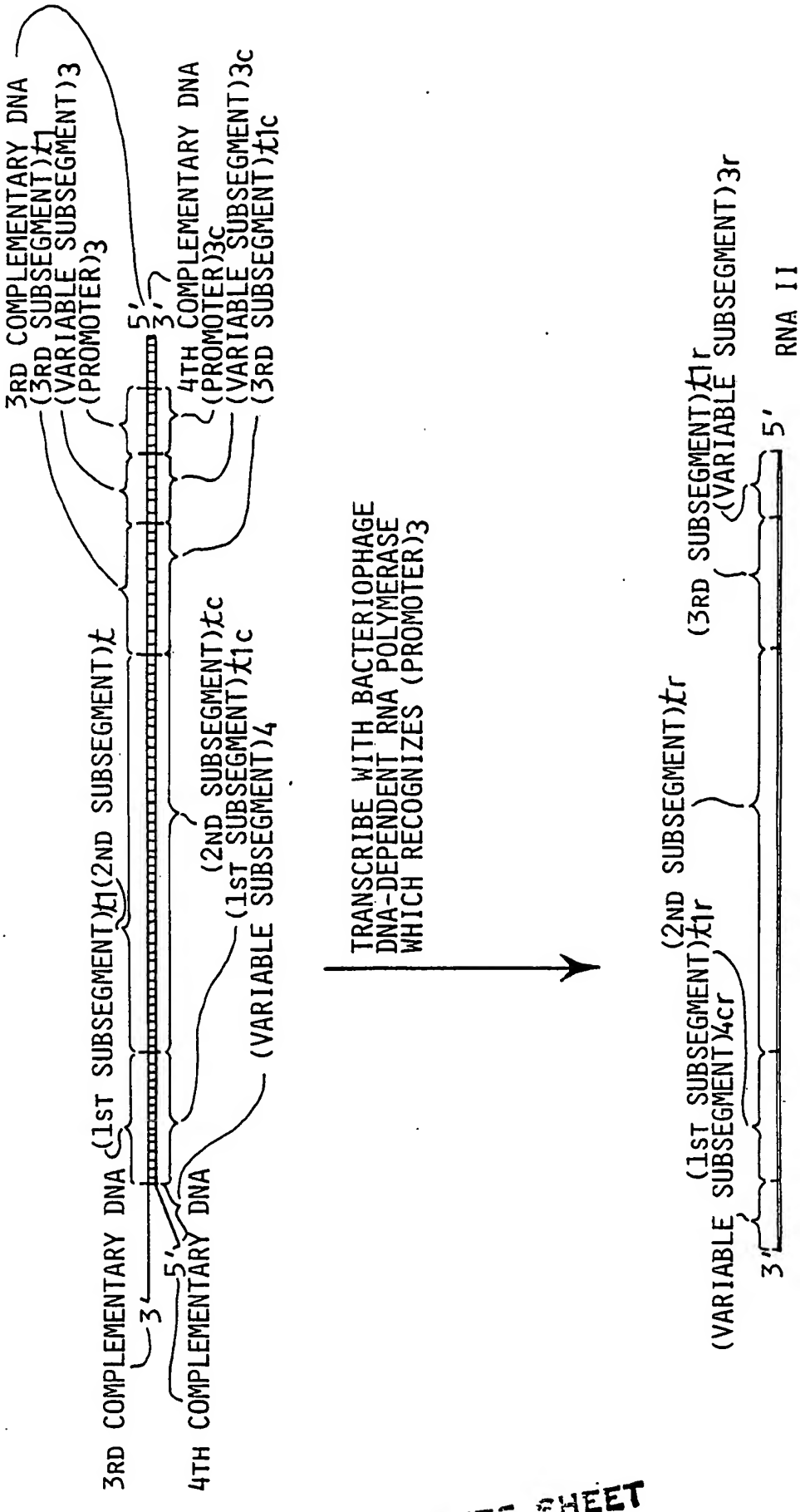


FIG. 2C

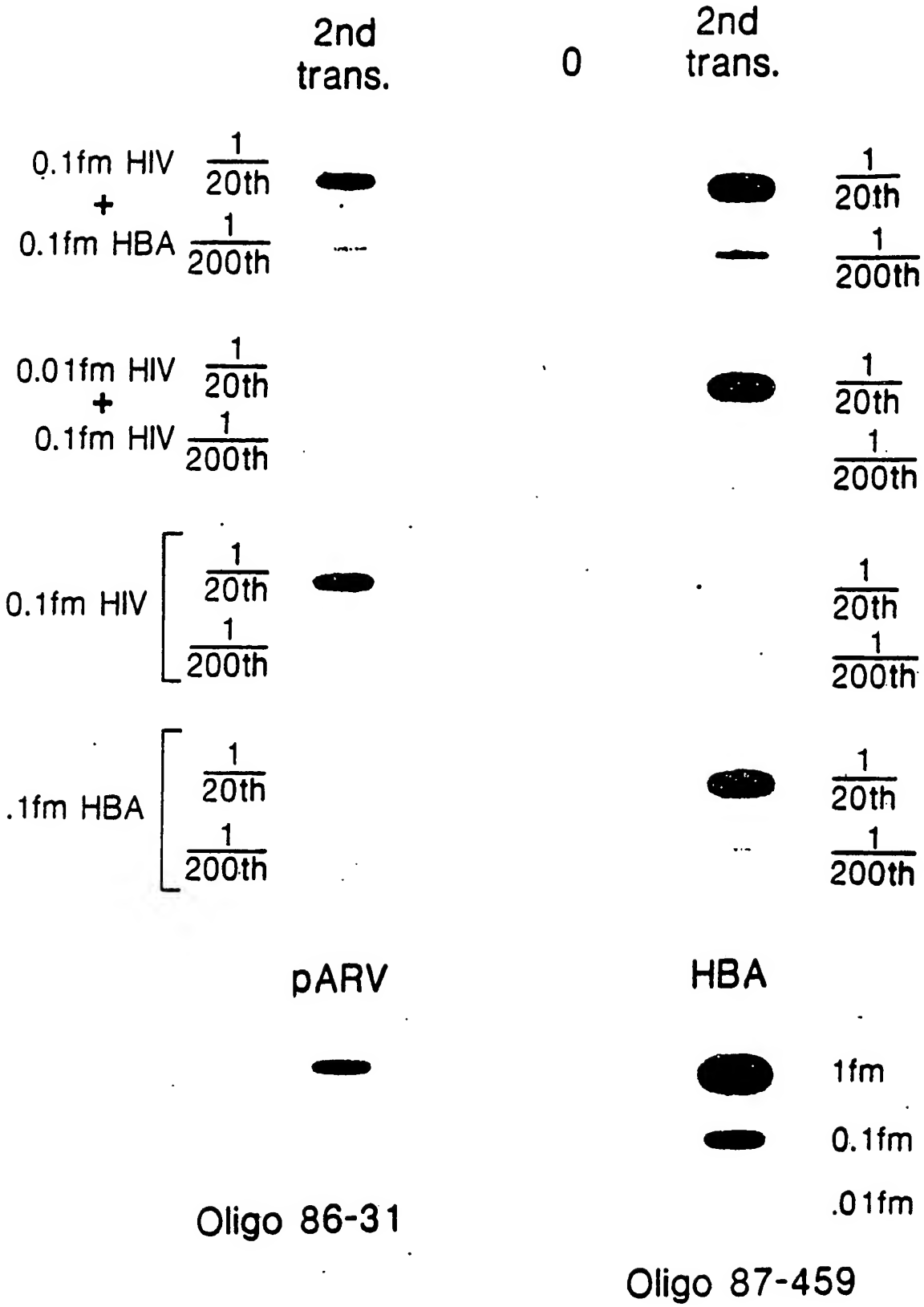
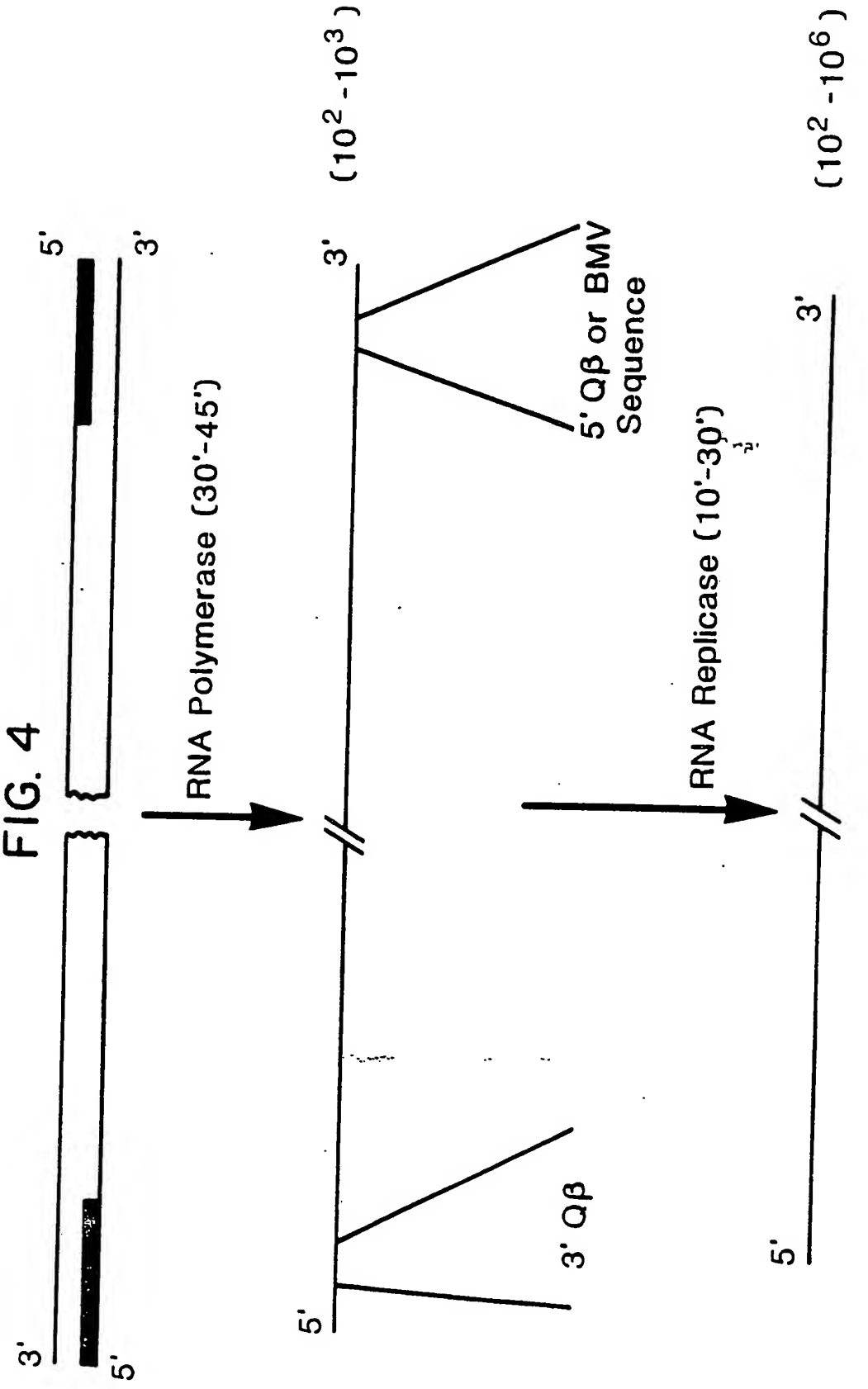

2nd trans.
0
2nd trans.
0.1fm HIV 1/20th + 0.1fm HBA 1/200th
0.01fm HIV 1/20th + 0.1fm HIV 1/200th
0.1fm HIV 1/20th 1/200th
.1fm HBA 1/20th 1/200th
1/20th
1/200th
1/20th 1/200th
1/20th 1/200th
1/20th
1/200th
pARV
HBA
1fm
0.1fm
.01fm
Oligo 86-31
Oligo 87-459

FIG. 3

FIG. 4


5'
3'
3'
5'
RNA Polymerase (30'-45')
3'
(10^2 -10^3)
5' Qβ or BMV Sequence
3' Qβ
5'
RNA Replicase (10'-30')
3'
(10^2 -10^6)
5'

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/02108

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 Q 1/68, C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 Q; C 12 N; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	Science, Vol. 239, p. 491-493; 29 January 1988 (E.S. STOFLET et al.): "Genomic Amplification with Transcript Sequencing" --	1-51
A	Nucleic Acids Research, Vol. 15, p. 8783-98, October 1987 (J.F. MILLIGAN et al.): "Oligoribonucleotid Synthesis using T7 RNA polymeras and synthetic DNA templates" --	1-51
A	Science, Vol. 230, p. 1350-5, 20 December 1985 (R.K. SAIKI et al.): "Enzymatic Aplification of B-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia" --	1-51
A	EP, A2, 0 201 184 (CETUS CORPORATION) 17 December 1986, whole document --	1-51

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19th October 1988	16 NOV 1988
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A2, 0 200 362 (CETUS CORPORATION) 10 December 1986 whole document --	1-51
P,A	EP, A2, 0 236 069 (CETUS CORPPRATION) 9 September 1987 p. 36-38 --	1-51
P,A	EP, A2, 0 229 701 (CETUS CORPORATION) 22 July 1987 whole document --	1-51

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

PCT/US88/02108
SA 23546

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 01/09/88
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0201184	12-11-86	EP-A- 0200362	05-11-86
		JP-A- 61274697	04-12-86
		JP-A- 62000281	06-01-87
		US-A- 4683202	28-07-87
		CA-A- 1237685	07-06-88
EP-A- 0200362	05-11-86	EP-A- 0201184	12-11-86
		JP-A 61274697	04-12-86
		JP-A- 62000281	06-01-87
		US-A- 4683202	28-07-87
		CA-A- 1237685	07-06-88
		US-A- 4683195	28-07-87
EP-A- 0236069	09-09-87	JP-A- 62240862	21-10-87
EP-A- 0229701	22-07-87	JP-A- 62217161	24-09-87